PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Eric N. OLSON *et al.*	Group Art Unit: 1632
Serial No.: 09/061,417	Examiner: M. Davis
Filed: April 16, 1998	Atty. Dkt. No.: MYOG:029US/SLH
For: METHODS AND COMPOSITIONS FOR THERAPEUTIC INTERVENTION IN CARDIAC HYPERTROPHY	

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October 18, 2004
Date

Steven L. Highlander

APPEAL BRIEF

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APPEAL BRIEF

Mail Stop Appeal Brief
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-01450

Dear Sir:

This brief is filed (in triplicate) in response to the Final Office Action mailed on March 18, 2004, regarding the above-captioned application. This brief was due on August 17, 2004, by virtue of the Notice of Appeal received by the PTO June 17, 2004. A request for a two-month extension of time to file an appeal brief is included herewith along with the required fee. This two-month extension will bring the due date to October 18, 2004 (since October 17, 2004 falls on a Sunday), which is within the six-month statutory period. Should such request or fee be deficient or absent, consider this paragraph such a request and authorization to withdraw the appropriate fee under 37 C.F.R. §§ 1.16 to 1.21 from Fulbright & Jaworski L.L.P. Account No.: 50-1212/MYOG:004USD1/SLH. Please date stamp and return the attached postcard as evidence of receipt.

I. Status of the Claims

Claims 1-40 were filed with the application. Claims 2, 3, 5-8, and 10-40 have been canceled. Thus, claims 1, 4 and 9 are pending, stand rejected, and are appealed herein. A copy of the pending claims are attached.

II. Status of the Amendments

No amendments have been submitted after issuance of the Final Office Action.

III. Statement of Interest

The real party in interest is the assignee, Board of Regents, University of Texas, and the licensee, Myogen, Inc. of Westminster, CO.

IV. Related Appeals and Interferences

There are no related appeals or interferences.

V. Summary of the Invention

The present invention involves a central mediator of cardiac hypertrophy and defines the molecular events linking calcium stimulation to cardiac hypertrophy. In particular, the present invention shows that Ca++ stimulation of the cardiac hypertrophic response is mediated through NF-AT3. Specification at page 4, lines 5-13. The invention provides methods of treating cardiac hypertrophy in a subject by inhibiting the function of NF-AT3 in a cardiomyocyte. Page 4, lines 5-10, 22-24. This method involves inhibiting the function of NF-AT3 and therefore relies on an interaction between an inhibitor and NF-AT3, thus the method also may comprise contacting the cardiomyocyte with an agent that binds to and inactivates NF-AT3. Page 4, lines 11-13, 19-20.

Furthermore, this agent may be a small molecule inhibitor or an antibody preparation. Page 4, lines 26-30; page 5, lines 1-2.

VI. Issues on Appeal

(i) Do claims 1, 4, and 9 satisfy the written description requirements of 35 U.S.C. §112, first paragraph?

(ii) Are claims 1, 4, and 9 enabled and not overly broad in scope under 35 U.S.C. §112, first paragraph?

(iii) Is claim 1 inherently anticipated under 35 U.S.C. §102(b) by Haverich *et al.* ("Haverich"; Exhibit A), Reid *et al.* ("Reid"; Exhibit B), McCaffrey *et al.* ("McCaffrey"; Exhibit C) and Martinez-Martinez *et al.* ("Martinez"; Exhibit D)?

VII. Grouping of the Claims

The claims stand or fall together.

VIII. Summary of the Argument

The examiner's written description rejection has repeatedly been maintained as the examiner alleges that there is a lack of a clear description of small molecule inhibitors of NF-AT3. The examiner primarily cites to the *The Regents of the University of California v. Eli Lilly* case (119 F.3d 1559, Fed. Cir. (1997)) for the proposition that "an adequate written description of a DNA requires a precise definition." Appellants submit that the examiner is attempting to create a rule of law from *Lilly* applied to non-DNA cases where none currently exists.

The rejection for lack of enablement draws specifically on MPEP §2164.03, requiring more detail from the specification to enable the claims. The examiner focuses heavily on his

perceived unpredictability in the art, and relies almost exclusively on the adage that a patent not require undue experimentation from one of skill in that art, both of which appellant traverses as incorrectly applied to the facts of this case. Finally, the examiner advances a standard for enablement that runs counter to established case law. In sum, the rejection is flawed, both legally and factually, and should be reversed.

Appellant has addressed the enablement issue by supplying supporting experimental evidence through an expert affidavit (Exhibit E), as well as pointing out that, for the disputed claims, the examiner has misapplied the undue experimentation standard. Reversal of the rejection is respectfully requested.

The prior art rejections are nothing more than an assemblage of references that discuss various unlinked elements of the claimed invention but never directly disclose the invention itself. No reference in and of itself contains the entire invention, and thus no reference actually anticipates the invention. The examiner relies on an inherency argument, where he has combined references that use cyclosporine (CsA) to combat transplantation disease with other references that show that CsA is also an NF-AT3 inhibitor. Still, the rejection must further assume that hypertrophy is usually associated with transplantation disease in order for these references to anticipate the use of an NF-AT3 inhibitor in treating hypertrophy. However, the examiner fails to show where the nexus between treating transplantation disease and treating hypertrophy is proven. It is then further assumed by the examiner that any drug that treats transplantation disease will by implication be treating hypertrophy. This assumption overlooks the fact that none of the clinical references even discuss hypertrophy. In sum, the rejection is based on a sketchy and poorly supported inherency argument, which fails the legal standard for anticipation. Thus, this rejection too should be reversed.

IX. Argument

A. Standard of Review

Findings of fact and conclusions of law by the U.S. Patent and Trademark Office must be made in accordance with the Administrative Procedure Act, 5 U.S.C. §706(A), (E), 1994. *Dickinson v. Zurko*, 527 U.S. 150, 158 (1999). Moreover, the Federal Circuit has held that findings of fact by the Board of Patent Appeals and Interferences must be supported by "substantial evidence" within the record. *In re Gartside*, 203 F.3d 1305, 1315 (Fed. Cir. 2000). In *In re Gartside*, the Federal Circuit stated that "the 'substantial evidence' standard asks whether a reasonable fact finder could have arrived at the agency's decision." *Id.* at 1312. Accordingly, it necessarily follows that an Examiner's position on Appeal must be supported by "substantial evidence" within the record in order to be upheld by the Board of Patent Appeals and Interferences.

B. Rejection Under 35 U.S.C. §112, First Paragraph (Written Description)

It is initially noted by appellants that the affidavits of Bush, Williams and Rothermel, submitted on July 9, 2003, and acknowledged and entered by the examiner in his final office action, were incorrectly filed with this case and are irrelevant to the arguments or discussion herein.

In regards to the case at hand, the examiner repeatedly asserts a written description rejection under 35 U.S.C. §112, first paragraph, alleging that there is a lack of a clear description of small molecule inhibitors of NF-AT3. The examiner primarily cites to the *Lilly* case for the proposition that "an adequate written description of a DNA requires a precise definition." Appellants submit that examiner is attempting to create a rule of law from *Lilly* where none currently exists. *Lilly* and its subsequent cases have not required that an invention must always

be specifically described as *Lilly* required for those particular DNA molecules, nor do the cases require that a genus must be described in its entirety, but rather that a genus may be claimed from a representative number of contained species.

Thus, *Lilly* is primarily applicable to the question of written description for as of yet unidentified DNA sequences or in cases where description of a species fails to properly extrapolate to the entire genus, such as where knowledge of one nucleotide sequence may be sufficient to describe a nucleotide sequence related only functionally to that first described sequence. Such situations are not presented in the case at hand, and the examiner's attempt to broadly extend *Lilly* to other scenarios when the invention is described through other terms or examples is not appropriate.

Recent cases elaborating on the holdings of *Lilly* show that "the failure of the patent to describe the claimed sequences by anything other than their function" is problematic, and that the proper standard varies depending on the invention and whether it can be described in more than a functional way. *See Enzo Biochem, Inc. v. Gen-Probe Inc.*, 285 F.3d 1013 (Fed. Cir. 2002). Both *Lilly* and *Enzo* require that "the disclosure must allow one skilled in the art to visualize or recognize the identity of the subject matter of the claim." The important point of both cases is that function alone, *i.e.*, wishful thinking, cannot support a set of claims to the molecules (or DNA) behind that function. However, it does not set up a proscription against the generic claiming of biological molecules. In this regard, it is thus of utmost importance to note that the present specification does not rely on function alone; specific examples and specific molecules are given so that one of skill in the art would be able to visualize or recognize the subject matter of the claims.

It is not now, nor has it ever been the law, that every aspect of a claimed invention need be present as physical examples for an invention to be described (this aspect of patent law has been upheld since the first cases in patent law, *see e.g. O'Reilly v. Morse*, 56 U.S. (15 How.) 62 (1853)). The examiner asserts, by way of example, that configuration of the second zinc finger of GATA4 is not known and that specific structures are not disclosed in the application for GATA4 mimetics, antisense molecules, or competitive inhibitors of NF-AT3, and that such lack of knowledge also somehow constitutes a failure of written description. Knowledge of the actual binding sites and exhaustive listings of structures are not a requirement for one of skill in the art to appreciate that the inventors had possession of the claimed invention. Moreover, as discussed in the declaration of Dr. Rick Gorczynski (Exhibit E), those of skill in the art would not doubt that GATA4 does indeed bind to NF-AT3, nor would they challenge the notion that interference with that interaction will have inhibitory effects on NF-AT3's ability to activate gene transcription of hypertrophic genes, such interference clearly being mediated by any of the molecules or agents listed above or referenced in the specification.

As stated in MPEP §2163, an "objective [of §112] is to put the public in possession of what the applicant claims as the invention." Appellants submit that, unlike the *Lilly* case, where the DNA molecules at issue had not yet been discovered, a number of the NF-AT3 targeting molecules disclosed by appellants are already known, and thus have been sufficiently described to put the public in possession of the invention. This provides yet another important distinction between the instant application and *Lilly* or *Enzo*, to which the examiner repeatedly points.

The written description requirement does not demand exhaustive listings of molecules and detailed description of binding sites and binding regions. Appellants again cite to *Lilly*, which states "a specification may, within the meaning of §112 P1, contain a written description

of a broadly claimed invention without describing all species that claim encompasses." This specification goes beyond simply claiming an undescribed molecule, it actually refers to GATA4 mimetics, DTC's, antisense molecules (p. 27, lines 12-20), antibodies, competitive inhibitors of NF-AT3 (p. 30, line 21) as well as other proteins that inhibit NF-AT3 (in addition to Examples 3, 6-9, see Summary of the Invention page 4, lines 15-25). These examples describe specific molecules known in the art and whose mention alone should be sufficient to satisfy the written description requirements of §112.

Furthermore, the examiner complains that antibodies and mimetics are described in a functional way and without detailed description of the actual binding sites. Just as above, appellants note that the examiner appears to be overextending the description requirements of *Lilly*. The specification describes specific molecules that interact with NF-AT3 in a way that goes beyond mere wishful thinking. The examiner argues that "the structure of the claimed GATA4 mimetics, [and] antisense molecules" is not disclosed. However, these molecules are defined by the prior art structures of the target molecules, and their absence from the specification is of no significance.

Further, these molecules are shown to interact, or can be proven with little experimentation to interact, with NF-AT3. That alone is sufficient to describe the invention in a comprehensible way to the public. The mere fact that they are not described atom by atom does not rob the claims of an adequate written description. The examiner also notes that the inhibitors have "no common structural attributes." However, appellants are unaware that commonality of structure is required for claiming a genus of inhibitors.

Finally, appellants point to the recent case of *Rochester v. Searle*, 358 F.3d 916 (Fed. Cir., (2004)), which states that "the patent specification [should] set forth enough detail to allow

a person of ordinary skill in the art to understand what is claimed and to recognize that the inventor invented what is claimed." The current claims call out methods of treatment by inhibiting NF-AT3 and the specification then describes both in words and examples a variety of ways to accomplish the claimed method. It is not a violation of written description that every possible mode for performing the invention is not subsequently supported by working examples, that has never been a requirement of patent law and is not a rule that is supported by any of the rulings regarding written description.

The above statements, taken in light of *Lilly* and *Enzo* as those courts intended, should successfully traverse the Examiner's rejections for lack of written description. Therefore, Appellants argue that this rejection should rightfully be reversed..

C. Rejection Under 35 U.S.C. §112, First Paragraph (Scope)

Claims 1, 4 and 9 are rejected for allegedly excessive "scope" under §112, first paragraph, although the rejection is a mere reiteration of a prior enablement rejection. The examiner has apparently rejected all of appellants' prior arguments, arguing that the art is unpredictable, that the invention has not been enabled by the specification, and that it would constitute "an undue burden" for one of skill in the art to practice the invention. Appellants have repeatedly argued and herein maintain that the examiner has misapplied the standard of undue experimentation.

It may be true that the use of NF-AT3 inhibitors to treat hypertrophy was not well-known at the time of filing, but the information provided in the specification, coupled with what was known prior to this invention, would allow one of skill in the art to practice the invention. The examiner's criticism almost seems to rise to the level of requiring a working model, a criticism

the examiner has consistently failed to address. According to MPEP §2164.02 "an applicant need not have actually reduced the invention to practice prior to filing." It is important to remember that "because only an enabling disclosure is required, [appellant] need not describe all actual embodiments. The absence of working examples will not by itself render the invention non-enabled. Furthermore, a single working example in the specification for a claimed invention is enough to preclude a rejection which states that nothing is enabled." MPEP §2164.02. Examples 6 through 9 clearly show *in vivo* proof that use of NF-AT3 inhibitors can be a method to treat hypertrophy. It is also not a requirement of the law, as the examiner asserts in regards to GATA4 mimetics, to teach how to make something that is readily understood by one of skill in the art. The specification describes the mimetic technology (see page 29, see also the cited reference of Johnson *et al.*, on same page) in a way sufficient for one of skill in the art to utilize that technology, which is the requirement of the law.

The examiner's rejections do not track any requirement of the laws of patents and go far beyond any currently accepted enablement or "scope" standards. Appellants refer to *In re Robins*, 429 F.2d 452 (CCPA 1970) cited by *Lilly*, stating, "Section 112 does not require that a specification convince persons skilled in the art that the assertions therein are correct." Furthermore, *Robins* holds that a "specification which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented must be taken as in compliance with the enabling requirement ... unless there is reason to doubt the objective truth of the statements therein." The *Robins* court also demands that "Section 112 requires nothing more than objective enablement. How such teaching is set forth, either by the use of illustrative examples or by broad terminology, is of no importance."

The examiner has also repeatedly stated that one of skill in the art would not know how to make and use the claimed method. *In re Wands*, 858 F.2d at 737 (Fed. Cir. 1988), states that so long as there is "considerable guidance" in the specification and "all of the methods to practice the invention [are] known," then "it would not require undue experimentation ... to practice the claimed invention." While more enablement may be required where the art is unpredictable, appellants repeat that there is no per se rule or requirement for a working model. The invention must simply enable one of skill in the art to practice that invention, and there is nothing contained in the current application that goes beyond the capabilities of one of skill in the art (MPEP §2164.01 - "the fact that experimentation may be complex does not necessarily make it undue, if the art typically engages in such experimentation."). Also instructive is *U.S. v. Telectronics, Inc.* 857 F.2d 778 (Fed. Cir. 1992), that "a patent need not teach, and preferably omits, what is well known in the art ... the test of enablement is whether one reasonably skilled in the art could make or use the invention from the disclosures in the patent coupled with information known in the art."

Furthermore, the Examiner repeatedly asserts that the entire field of antisense technology was unpredictable and unreliable as of the time of filing. He uses as his proof of point a reference from 1995 by Gura (*Science*, 1995, 270:575-577; Exhibit F), even though the application was filed in 1998, and the examiner now claims as supportive evidence a reference cited in appellants' last response (Bennett, *Biochem. Pharmacol.*, 1998, 55(1):9-19; Exhibit G). Bennett states that "antisense oligonucleotides are widely used as tools to explore the pharmacological effects of inhibiting expression of a selected gene product," and even more importantly, that "with careful selection, proper controls, and careful dose-response curves it is possible to utilize antisense oligonucleotides as effective research tools and potentially as

therapeutic agents." The examiner is convinced that "potentially" supports his case that antisense is non-enabled technology and ignores the explicit teaching of the reference that antisense is a viable and utilizable technology according to the authors.

Appellants thus reassert that the specification does, in fact, enable one of skill in the art to practice the claimed invention. Therefore, it is respectfully requested that the claims be reconsidered and the rejection be reversed.

D. Rejection Under 35 U.S.C. §102

Claim 1 has been repeatedly rejected by the examiner under 35 U.S.C. §102(b) as allegedly being anticipated by Haverich *et al.*, Ried *et al.*, McCaffrey *et al.*, and Martinez-Martinez *et al.* (Exhibits A, B, C and D respectively). Appellants have asserted before, and again restate, that for literal anticipation of a claim, "a reference must disclose every element of the challenged claim and enable one skilled in the art to make the anticipating subject matter." *PPG Industries Inc. v. Guardian Industries Corp.*, 37 USPQ.2d 1618, 1624 (Fed. Cir. 1996). The examiner appears to have misread the claims at issue, as the examiner asserts that the method is to treatment of a cardiomyocyte, when clearly claim 1 is directed to a method of treating cardiac hypertrophy. Therefore, if the prior art does not disclose treatment of hypertrophy, it cannot be used to support an inherency rejection.

In light of the above statement, appellants maintain their prior assertion that every element in claim 1 is not found in any of the prior art references supplied by the examiner. Claim 1 teaches treatment of hypertrophy by inhibiting the function of NF-AT3 in a cardiomyocyte using a compound that inhibits the function of NF-AT3. The Haverich and Reid references teach the use of cyclosporin A (CsA) for treatment of transplantation disease; they do

not teach, much less suggest treatment of hypertrophy or effects on cardiac structure. They are instead directed towards improving cardiac function in a post-transplant environment. Additionally, while the McCaffrey and Martinez-Martinez references do teach that CsA is an NF-AT3 inhibitor, they do not teach the use of an NF-AT3 inhibiting compound to treat hypertrophy. Not one of these references teaches the invention, nor does the collection inherently predict, much less describe the invention. Not once in any of these references can the words "cardiac hypertrophy" be found and, furthermore, there is not a single reference that states that all transplantation disease is accompanied by, foreshadows or leads to cardiac hypertrophy. Therefore, the examiner cannot properly assert that the claimed method has been practiced before in these references. Thus, they fail to meet the criteria required by the law to suffice as anticipatory references for an inherency rejection.

The Examiner has also pointed to *Ex parte Novitski*, 26 U.S.P.Q.2D (BNA) 1389 (BPAI, 1993), to support an inherency argument, and argues that appellants have not addressed this citation. This is untrue. *Novitski* merely states that inherent anticipation may lie, that claims are interpreted as broadly as reasonably possible, and that limitations are not read into the claims. However, contrary to *Novitski*, a limitation of the instant claims is expressly stated as treating cardiac hypertrophy. Thus, while nothing must be read into the claims, the claims cannot be read to exclude this limitation.

Appellants submit that the case law requires that an inherent disclosure "must be certain." *Ex parte McQueen*, 123 USPQ 37 (Bd. App. 1958). There is no evidence from the cited references that hypertrophy had been treated or even analyzed. The prior art specifically deals with transplantation disease and cardiac function after transplant in response to CsA application. Transplantation disease has not and is not defined as cardiac hypertrophy, and it is possible to

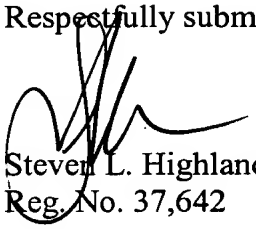
have one without the other, thus, there cannot be any inherency. The references do not teach a treatment for hypertrophy nor would one of skill in the art be expected to infer from these references that CsA, and subsequently NF-AT3 inhibitors, were being used to treat hypertrophy. The examiner has not even attempted to address this issue, instead merely repeating the previous rejections.

Thus, again, appellants submit that in the absence of an indication that cardiac hypertrophy was in fact treated in the work described by the cited references, the rejection cannot be certain and therefore fails to meet the standards required for an inherency rejection under 35 U.S.C. §102(b). Appellants therefore respectfully request that the rejection under §102(b) be reversed.

X. Conclusion

In light of the foregoing, appellants respectfully submit that all pending claims are definite and supported by the application as filed. Therefore, it is respectfully requested that the Board reverse each of the pending rejections.

Respectfully submitted,



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Date: October 18, 2004

APPENDIX 1: LISTING OF CLAIMS

1. A method of treating hypertrophy in a subject comprising the step of inhibiting the function of NF-AT3 in a cardiomyocyte, wherein the inhibition of NF-AT3 function inhibits hypertrophic gene expression, thereby treating hypertrophy.

2-3. (Canceled)

4. The method of claim 1, wherein inhibiting the function of NF-AT3 comprises contacting said cardiomyocyte with an agent that binds to and inactivates NF-AT3.

5-8 . (Canceled)

9. The method of claim 4, wherein the agent that binds to and inactivates NF-AT3 is an antibody preparation or a small molecule inhibitor.

10-40. (Canceled)

APPENDIX 2: CITED AUTHORITIES

Administrative Procedure Act, 5 U.S.C. §706(A), (E), 1994

Dickinson v. Zurko, 527 U.S. 150, 158 (1999)

Enzo Biochem, Inc. v. Gen-Probe Inc., 285 F.3d 1013 (Fed. Cir., 2002)

In re Gartside, 203 F.3d 1305, 1315 (Fed. Cir., 2000)

Manual of Patent Examining Procedure §2163

Manual of Patent Examining Procedure §2164.01

Manual of Patent Examining Procedure §2164.02

Manual of Patent Examining Procedure §2164.03

In re Marzocchi, 169 USPQ 370 (CCPA, 1971)

Ex parte McQueen, 123 USPQ 37 (Bd. App., 1958)

Ex parte Novitski, 26 U.S.P.Q.2D (BNA) 1389 (Bd. Pat. Apps., 1993)

O'Reilly v. Morse, 56 U.S. (15 How.) 62 (1853)

PPG Ind. Inc. v. Guardian Industries Corp., 37 USPQ.2d 1618, 1624 (Fed. Cir., 1996).

The Regents of the University of California v. Eli Lilly, 119 F.3d 1559, (Fed. Cir., 1997)

In re Robins, 429 F.2d 452 (CCPA, 1970)

Rochester v. Searle, 358 F.3d 916 (Fed. Cir., 2004)

U.S. v. Telectronics, Inc. 857 F.2d 778 (Fed. Cir., 1992)

In re Wands, 858 F.2d at 737 (Fed. Cir., 1988)

APPENDIX 3: EXHIBITS

Cyclosporin A and Transplant Coronary Disease After Heart Transplantation: Facts and Fiction

A. Haverich, A. Costard-Jäckle, J. Cremer, G. Herrmann, and R. Simon

TRANSPLANT coronary disease (TCD) remains the single most important risk factor of death in heart transplant recipients surviving beyond the first postoperative year. Many clinical variables have been associated with the development of this complication, including older age of donor hearts, poor HLA matching, hyperlipidemia, hypertension, and others. Recently, chronic application of cyclosporin A (CyA) has been suggested to also represent a risk factor for TCD. This association was constructed on the basis of animal experiments, where significant impairment of endothelial and smooth muscle cell function was seen under CyA therapy.

THE PROBLEM

In our own series covering more than 400 heart transplants over a period of 8 years, TCD occurred in 11.2% of patients after 1 year, 17.9% after 2 years, and 28.6% after 3 years (Fig 1). In general, these coronary changes were asymptomatic and only detected by angiography. The linearized rate of yearly detection does reflect the experience of other larger transplant programs, which also report an annual occurrence of about 10%.

In our series as well as in others, TCD does have a significant impact on late death. As such, 62% of all fatalities beyond the first posttransplant year were directly related to TCD. A similar prevalence was observed in other series.[1] Graft dysfunction and rejection do account for more than 50% of late deaths in the registry of the International Society for Heart and Lung Transplantation.[2]

Considering the magnitude of the problem, intense search for risk factors precipitating TCD appears to be more than justified. Among many others, CyA treatment has been identified to cause impaired vasomotion, potentially resulting in TCD.

ANIMAL EXPERIMENTS

A large number of animal experiments have been conducted recently with the aim of understanding the pathomechanisms involved in CyA treatment and subsequent vessel wall dysfunction. Most studies were done using rings of the descending thoracic aorta in rats.

In one of these series, Auch-Schwelk et al[3] applied 60 mg/kg/d of CyA for 6 weeks before studying the aortic rings. At the time of harvesting, mean CyA blood level was 3376 ± 472 μg/L. Under these conditions, they were able to identify significantly diminished properties of endothelial and smooth muscle cell function in the aorta. Both endothelium-dependent and -independent vasodilation were severely impaired as demonstrated by the application of acetylcholine and nitroprusside sodium. Vasoconstriction induced by application of norepinephrine and potassium chloride, by contrast, was significantly enhanced. An identical experimental design applied by the same group in a subsequent study[4] showed that these impaired functional characteristics were reversible if the calcium channel blocking agent verapamil was given.

Rego et al[5] in a similar experiment could not find any significant changes in the vasomotor response of aortic rings following chronic application of CyA at a dose of 5 mg/kg/d. With administration of 10 mg/kg/d, by contrast, he could show the same alterations in constrictive and dilative properties of the rat aorta. Importantly, in their experiments, this pathologic response was reversible upon cessation of CyA therapy for 10 days.

Reversibility of changes was also noted in experiments by Xue et al,[6] who showed phentolamine to significantly reduce the pathologic response of CyA (dose 15 mg/kg/d)-treated rat aorta. Similarly, Gallego et al[7] identified L-arginine as a potent measure to counteract changes in aortic vasomotion following CyA application at very high doses (25 mg/kg/d). Their hypothesis of lack of endothelial-derived relaxing factor (EDRF) release in such vessels would be supported by the findings obtained after addition of the nitricoxyde (NO) donor L-arginine. This substance also reduced the abnormally increased calcium uptake in such aortic rings.

FINDINGS IN HUMANS

To compare these findings in animals with clinical data, O'Neil et al[8] performed an extremely attractive clinical experiment. They studied the endothelium-dependent and -independent vasodilation of human coronary arteries in vitro. Although the majority of specimens were normal vessels obtained from heart transplant recipients, three coronary arteries were taken from long-term CyA-treated patients at the time of heart-lung retransplantation for chronic lung rejection. Here, a completely normal response both to nitroglycerin and substance P was observed, even after 37 months of immunosuppression using CyA. They therefore concluded that CyA would not impair EDRF release even when applied long term.

From the Departments of Cardiovascular Surgery (A.H., A.C.-J., J.C.) and Cardiology (G.H., R.S.), University of Kiel, Kiel, Germany.

Address reprint requests to A. Haverich, Department of Cardiovascular Surgery, University of Kiel, Kiel, Germany.

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Transplantation Proceedings, Vol 26, No 5 (October), 1994: pp 2713–2715

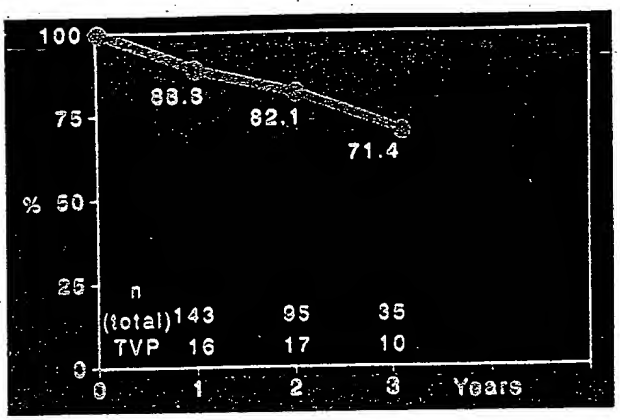

Fig 1. Linearized rate of freedom from TCD up to 3 years after orthotopic transplantation.

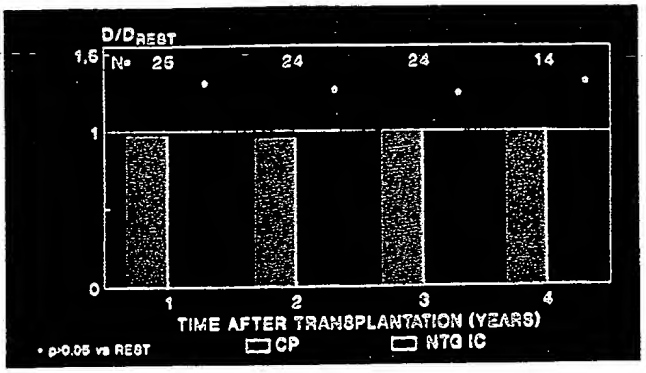

Fig 3. Response to nitroglycerin (NTG) in human coronary arteries 1 to 5 years after heart transplantation. Note significant increase in coronary artery diameter at all time intervals.

These data would support our own results on substance P—it triggered NO release in long-term surviving patients after heart transplantation. We were able to demonstrate a normal coronary artery response to substance P up to 5 years after orthotopic transplantation.[9] In patients with TCD, however, coronary vasodilation was significantly impaired (Fig 2). In addition, the Kiel group showed a continual normal response of coronary vasodilation following application of nitroglycerin and papaverine during yearly coronary angiography studies up to 4 years after transplantation (Fig 3).

There is further evidence that CyA may not be an independent risk factor for TCD. Five years ago, Gao et al[10] published long-term results of two cohorts of patients after heart transplantation. One group received conventional therapy including azathioprine (without use of CyA), the other group was maintained on a CyA-based immunosuppressive protocol. Linearized rates of freedom from TCD at 1, 3, and 5 years were 90, 70, and 58% in the azathioprine group and 93, 69, and 50% in the CyA group, respectively, without significant differences at the given intervals (Table 1). These data do reflect our own experience with an annual risk of about 10% of patients with de novo development of TCD (Fig 1). These findings neither support the concept of severely impaired coronary vasomotion in human heart transplant recipients on long-term treatment of CyA nor suggest that this drug promotes TCD by the mechanism of impaired vasomotion.

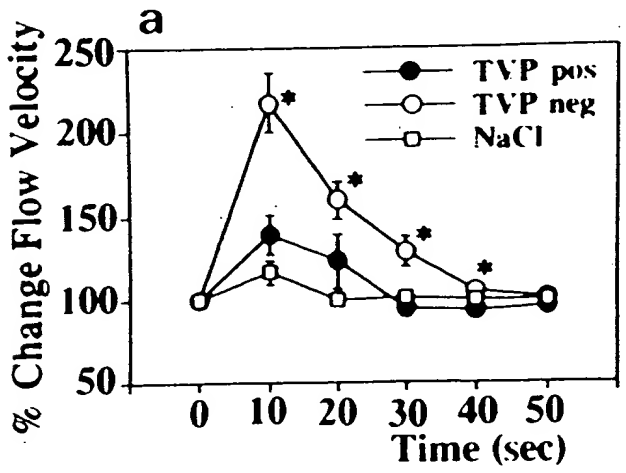

Fig 2. Effect of intracoronary application of substance P on flow velocity in heart transplant recipients with and without transplant vasculopathy (TVP).

SUMMARY AND CONCLUSIONS

From published data in the currently available literature on animal experiments, it can be concluded that CyA may be involved in the development of hypertension and renal dysfunction. These side effects are probably related to a disturbed vasomotor tone and a pathologic reaction to physiologic vasoconstrictive and vasodilative agents following administration of high doses of CyA. These effects are dose related and reversable and were undetected at clinically applied levels of immunosuppression. It therefore appears to be unlikely that this pathologic response is operative in precipitating or promoting TCD, which is supported by findings of in vivo and in vitro studies on human coronary arteries as well as by comparison of immunosuppressive protocols comparing series with and without application of CyA.

Chronic graft dysfunction, as depicted by late mortality in heart and isolated lung transplant recipients and by loss of kidney allograft survival, yields an annual rate of between 4

Table 1. Freedom From TCD at 1, 3, and 5 Years after Heart Transplantation Using Azathioprine (Aza)- and CyA-Based Immunosuppression

	Aza (%)	CyA (%)	p
1 y	89	86	n.s.
3 y	74	63	n.s.
5 y	58	50	n.s.

Note. There were no significant differences between groups (from Gao et al[10]).

and 6% per year. It is only in heart transplantation that TCD has been tried to be associated with CyA application. If CyA-related damage were to occur in coronary arteries independent from immunologic factors, the incidence of coronary disease should be similar in heart, lung, liver, and kidney transplants. This is not the case.

Although continuous investigation on the side effects of immunosuppressive agents is definitely necessary, we should concentrate our research activities on potential prophylactic and therapeutic measures to overcome the single most important risk factor of long-term surviving patients after solid organ transplantation: chronic rejection.

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Br Heart J 1988;59:397–402

)eterminants of left ventricular function one year ter cardiac transplantation

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MMARY Left ventricular systolic function was assessed by radionuclide angiography in 107 isecutive transplant recipients who were alive one year after operation. Mean (SEM) ejection ction was 62·4 (4·6) at rest and 68·8 (5·4) on exercise. The influence of donor-related factors nor age and sex, ischaemia time), recipient-related factors (recipient age and sex, frequency of te rejection), type of immunosuppression (cyclosporin/azathioprine or prednisolone/azath-rine), and frequency of hypertension on left ventricular function one year after operation was mined by univariate and multivariate analysis. There was a close association both at rest and on rcise between a higher ejection fraction and treatment with cyclosporin/azathioprine. There a trend for lower donor and recipient age, shorter ischaemia time, and fewer rejection episodes e associated with better left ventricular function, but this was not statistically significant.
eft ventricular systolic function was well maintained in most patients a year after cardiac isplantation. The type of immunosuppression used had a strong influence on the left ventricular tolic function of the transplanted heart.

rdiac transplantation has become established as an ctive form of treatment for patients with severe ocardial disease. The one year survival is 75–%[1–4] and the quality of life of most of these patients ood. The late results of the operation are as yet not wn. One of the main factors determining the ger term outcome of transplantation is left ven-ular function, which can be influenced by several ors related to the donor or recipient or to the type munosuppression used.
'e have examined left ventricular function one r after transplantation and have assessed the sible influence of some of these factors.

ents and methods

Y GROUP

studied 107 consecutive recipients who were one year after transplantation and who had rgone operation between February 1980 and mber 1984 (fig 1). Four patients were not ble for study because they lived abroad. There were 101 men, and the age range of the group at the time of operation was 12–59 (mean 44·1) years. An orthotopic operation had been performed in 93 (87%) patients and a heterotopic one in 14 (13%).

During this period, 155 patients had cardiac transplantation. There were 44 early (<1 year) deaths, 19/39 (48·7%) of those receiving prednisolone and azathioprine and 25/116 (21·5%) of those on cyclosporin/azathioprine. The late mortality was 9/20 (45%) in the prednisolone and azathioprine group (follow up 15–88 (mean 49·5) months) and 4/91 (4·4%) in the cyclosporin and azathioprine group (follow up 22–57 (mean 36·6) months).

RADIONUCLIDE VENTRICULOGRAPHY

Left ventricular function was assessed by measurement of ejection fraction with gated radionuclide cineangiography at rest and on exercise. Red blood cells were labelled in vivo with 15 mCi technetium-99m. Images were acquired with an all purpose parallel-hole collimator and a gamma scintillation camera (Ohio-Nuclear). Studies were performed with the patient at rest in the supine position in a 40° left anterior oblique projection. Symptom limited exercise was performed in a semi-supine position on a bicycle ergometer with an initial

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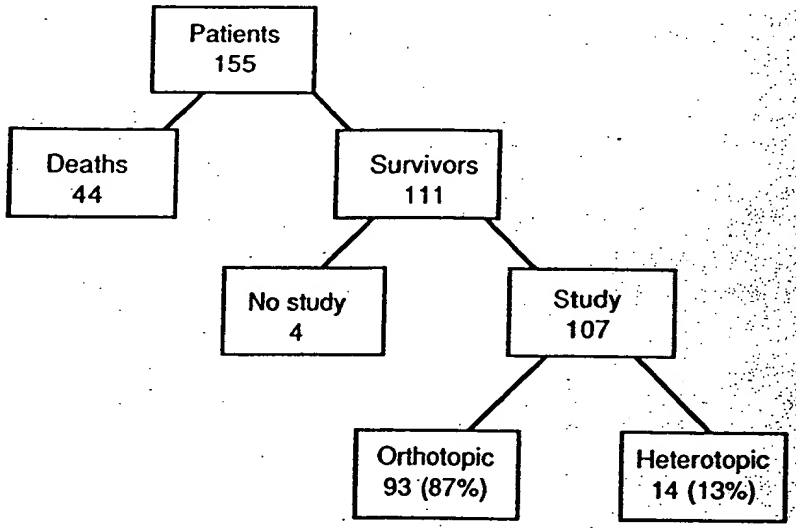


Fig 1 *Study population of patients who had cardiac transplants between February 1980 and December 1984.*

workload of 25 W and data were acquired over 10 minutes once a steady state had been reached. The ejection fraction was calculated by the multiple regions of interest method with computer-determined background areas. Exercise studies were not performed in heterotopic recipients in view of the difficulty in obtaining reliable electrocardiographic gating of the donor heart.

VARIABLES

Donor related

Donor age and sex.—Figure 2 shows the age distribution of the donors. Donors were aged from 9 to 40 (mean 21·7) years. There were 50 (47%) donors below the age of 20, 38 (35%) between 20 and 30, 18 (17%) between 30 and 40, and 1 (1%) aged 40. Seventy nine (74%) were male and twenty eight (26%) female.

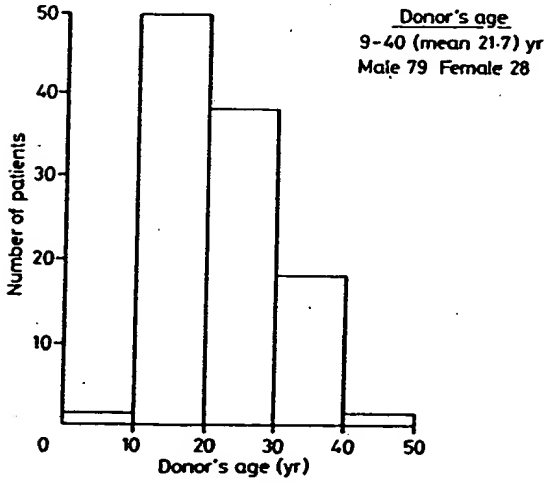


Fig 2 *Distribution of ages of donors.*

Ischaemia time.—The duration of the ischaemia time, defined as the time between clamping of the donor aorta and reperfusion of the donor heart in the recipient, varied from 70 to 300 (mean 138) minutes. Table 1 shows the variation in ischaemia time.

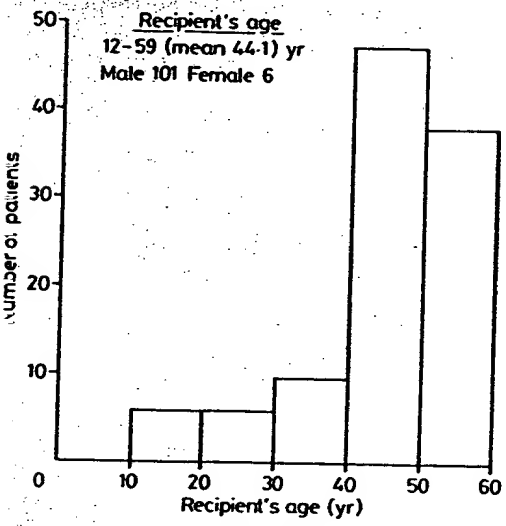
Recipient related

Recipient age and sex.—The age range of the recipients was 12–59 (mean 44·1) years (fig 3). Eighty five (79%) patients were aged ≥ 40 years at the time of transplantation. One hundred and one (94%) recipients were male.

Acute rejection.—Table 2 shows the number of episodes of acute rejection in each patient in the first year after operation. A rejection episode was defined as one during which an endomyocardial biopsy

Table 1 *Distribution of ischaemia time of donor hearts*

Ischaemia time (min)	*No donors (%)*
60– 90	7 (6)
90–120	36 (34)
120–150	25 (24)
150–180	15 (14)
180–210	12 (11)
210–240	8 (7)
240–300	4 (4)



3 *Distribution of ages of recipients.*

cimen showed infiltration of interstitial, perivas-ar, or endocardial pyroninophilic mononuclear ls with or without myocytolysis. Patients were ted with methylprednisolone (1 g daily) for three s and/or antithymocyte globulin. The mean num- of rejection episodes per patient during the first r after operation was 2·7.

unosuppression

ombination of oral steroids (prednisolone 1·5 mg/ day, reducing to 0·5 mg/kg/day) and azathioprine g/kg/day) was used as immunosuppression in the t 19 (18%) patients in this study.

The next 88 (82%) patients were given a com-ation of cyclosporin (5–20 mg/kg/day) and thioprine (2 mg/kg/day). Oral steroids were not tinely used in these patients.

ertension

ertension was defined as a blood pressure read-≥145/90 on two or more occasions during the year. In the prednisolone and azathioprine p 2/19 (11%) were hypertensive and in the sporin and azathioprine group 24/55 (44%) hypertensive.

ardial preservation

omas' cardioplegic solution, 20 ml in 1 litre of l saline introduced into the aortic root, was or donor heart preservation in all cases. The were then transported in Hartmann's solution

Table 2 *Occurrence of acute rejection in first year after transplantation*

No of episodes	*No of patients*	*%*
0	10	9
1	20	18
2	17	16
3	35	33
4	6	6
5	11	10
6	6	6
7	1	1
8	1	1

Statistical analysis

Data were analysed by simple and multiple linear regression. The regression coefficient was obtained and p value was determined. $P < 0.05$ was regarded as significant.

Results

LEFT VENTRICULAR FUNCTION

The distribution of ejection fraction in the study group at rest (fig 4) and on exercise (fig 5) is shown. The ejection fraction was 60% in 75 (70%) patients at rest and 79% patients on exercise. Left ventricular function was impaired (ejection fraction <50%) in 12 (11%) patients at rest and three patients on exercise.

The mean (SEM) ejection fraction in a group of normal volunteers in our laboratory was 63 (3·0) % at rest and 71 (2·3) % on exercise. Pooled data on ejection fractions in the normal population from 28 centres world wide gave a value of 62·3 (6·1) % at rest and 70·3 (7·6) % on exercise.[5]

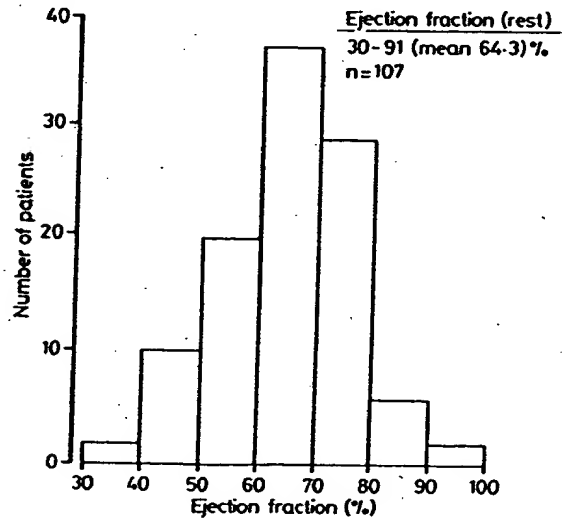


Fig 4 *Range of ejection fraction of study group at rest.*

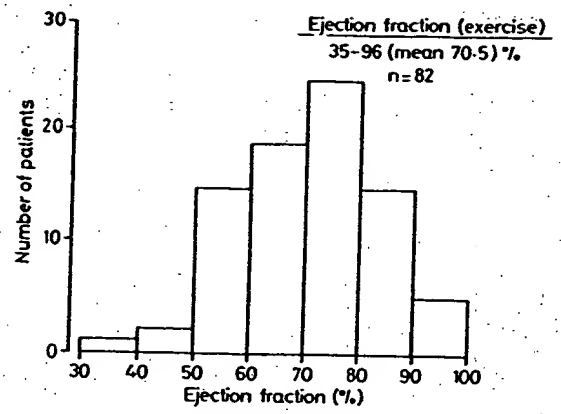


Fig 5 *Range of ejection fraction of 82 patients undergoing exercise studies.*

UNIVARIATE ANALYSIS (TABLE 3)

There was a significant association between treatment with cyclosporin and azathioprine and a higher ejection fraction at rest ($p < 0.01$) and on exercise ($p < 0.001$). Greater recipient age was associated with a lower ejection fraction at rest ($p < 0.01$) but not on exercise.

There was a trend for lower donor age, smaller number of rejection episodes, and shorter ischaemia time to be associated with improved ejection fraction both at rest and on exercise but the 95% confidence limit for this trend was not statistically significant.

MULTIVARIATE ANALYSIS (TABLE 4)

With multivariate analysis the association between treatment with cyclosporin/azathioprine and higher ejection fraction at rest and on exercise persisted ($p < 0.05$ and $p < 0.01$ respectively). No significant association was demonstrated between resting or exercise ejection fraction and the other variables.

Table 3 *Evaluation of influence of variables on rest and exercise ejection fraction by univariate analysis*

	Regression coefficient	*t value*	*p*
	Rest		
Cyclosporin	7·84	2·71	< 0·01
Recipient's age	−0·25	−2·31	< 0·01
Donor's age	−0·28	−1·70	< 0·10
Acute rejection	−1·05	−1·61	< 0·50
Donor's sex (M)	−3·40	−1·34	< 0·50
Ischaemia time	−0·16	−0·66	> 0·50
Recipient's sex (M)	2·39	0·48	> 0·50
	Exercise		
Cyclosporin	11·51	3·46	< 0·001
Recipient's age	−0·08	−0·60	> 0·50
Donor's age	−0·29	−1·38	< 0·50
Acute rejection	−1·05	−1·37	< 0·50
Donor's sex (M)	2·30	0·73	> 0·50
Ischaemia time	−0·03	−0·86	< 0·50
Recipient's sex (M)	1·41	0·24	> 0·50

Table 4 *Multivariate analysis of influence of variables on left ventricular function (ejection fraction)*

	Regression coefficient	*t value*	*p*
	Rest		
Cyclosporin	6·58	2·13	< 0·05
Recipient's age	−0·20	−1·71	< 0·10
Donor's age	−0·12	−0·69	< 0·50
Acute rejection	−0·66	−1·00	< 0·50
Donor's sex (M)	−3·10	−1·24	< 0·50
Ischaemia time	−0·03	−1·36	< 0·50
Recipient's sex (M)	−1·71	−0·34	> 0·50
	Exercise		
Cyclosporin	11·36	3·08	< 0·01
Recipient's age	0·01	0·01	> 0·50
Donor's age	−0·16	−0·72	< 0·50
Acute rejection	−0·31	−0·40	> 0·50
Donor's sex (M)	2·47	0·80	< 0·50
Ischaemia time	−0·03	−1·04	< 0·50
Recipient's sex (M)	0·64	0·11	> 0·50

SUBGROUP ANALYSIS

Table 5 shows an examination of factors relating to patients with the lowest (group A) and highest (group B) ejection fractions.

Analysis of results confirmed that there is no significant difference between the distribution of factors apart from the type of immunosuppression used (table 6).

IMMUNOSUPPRESSION: DISTRIBUTION OF VARIABLES

Figure 6 shows the distribution of variables in the two immunosuppression treatment groups.

Discussion

Cardiac transplantation has become an effective therapeutic option in the management of end stage heart disease and can be performed with a low operative mortality and early postoperative mortality. The success of the procedure, however, will be determined by its ability to maintain normal cardiac performance both at rest and on exercise in the long term.

In this study, we measured left ventricular ejection fraction determined by radionuclide angiography because this technique is non-invasive and provides some information about changes on exercise. These variables, however, in common with all ejection phase indices, suffer the disadvantage of being load-dependent. In a previous study we showed that after transplantation, the inotropic state of the left ventricle determined by load-independent indices was normal.[6] But this form of assessment of ventricular

ble 5 *Distribution of variables in patients with lowest ejection fractions (group A) and highest ejection fractions oup B)*

e No	EF% Rest Ex	Recipient's age/sex	Donor's age/sex	Acute rejection	Ischaemia time	Immuno-suppression
oup A:						
	32 (40)	47 M	25 M	6	110	Pred/Aza
	39 (35)	19 F	30 M	6	190	Cya/Aza
	40 (—)	48 M	39 F	4	150	Cya/Aza
	41 (55)	46 M	16 M	3	115	Cya/Aza
	42 (—)	54 M	20 F	3	255	Cya/Aza
	43 (55)	51 M	24 M	3	115	Cya/Aza
	44 (—)	46 M	23 F	3	90	Pred/Aza
	45 (54)	50 M	18 M	3	128	Pred/Aza
ıp B:						
	91 (95)	36 F	22 M	3	99	Cya/Aza
	91 (90)	43 M	32 F	0	120	Cya/Aza
	88 (96)	14 M	9 M	3	125	Cya/Aza
	88 (95)	53 M	35 M	5	120	Cya/Aza
	83 (85)	44 M	18 M	1	157	Cya/Aza
	83 (—)	34 M	36 M	0	239	Cya/Aza
	82 (79)	44 M	26 M	5	80	Cya/Aza
	80 (80)	48 M	19 M	4	139	Cya/Aza

azathioprine; Cya, cyclosporin; Pred, prednisolone.

ction could not be applied to the large numbers of ents in the current study and therefore the two lies cannot be compared directly.

7e found that in most cases the systolic function of transplanted heart, as determined by left ven-ılar ejection fraction at rest and on exercise, was maintained one year after operation. These ings accord with other studies in which radio-ide angiography was used[7 8] and with data from iac catheterisation one year after transplanta-[9 10] Contrary to the findings of Devineni *et al* saw no increase in ejection fraction on exercise -tients treated with cyclosporin,[11] we noted a ficant increase in ejection fraction on exercise ·(1·4)%, compared with resting values ·1·2)%) in the cyclosporin treated group. These ts were significantly better than for those in nts treated with conventional immunosuppres-(61·1(2·5)%, 57·7(2·7)% respectively).

: found that the only factor that was significantly iated with improved left ventricular function at rest and on exercise was treatment with sporin. The cyclosporin and azathioprine ıts were significantly younger (43·4 *vs* 47·3 yr) and had fewer episodes of acute rejection (2·50 *vs* 3·73) in the first year after transplantation than patients given prednisolone and azathioprine. In addition, their left ventricular function was better despite a significantly longer ischaemia time (142·5 *vs* 118·2 min) and a higher frequency of hypertension (43·6% *vs* 10·5%). These factors assessed individually were not shown to exert significant influence upon ventricular function although there was a trend for younger donor and recipient age and, to a lesser extent, shorter ischaemia time and smaller number of rejection episodes to have a favourable influence on left ventricular function. Therefore, the mechanism by which cyclosporin exerts its beneficial effect on systolic function has not been defined. Despite examination of the distribution of variables in patients with the highest and lowest ejection fractions, no factor other than the type of immuno-suppression used was identified as being an important determinant of ventricular function.

This work is part of a continuing programme to evaluate the long term results of cardiac transplantation. It is hoped that the information provided will help in optimising the results of this procedure.

6 *Comparison of ejection fraction (mean (SEM)) and values of variables (mean (SEM)) between group A and* B

EF%	Recipient's age*	Donor's age*	Acute rejection*	Ischaemia time*	Immuno-suppression
40·7 (1·44)	45·2 (3·80)	24·4 (2·60)	3·87 (0·48)	144·1 (19·2)	Cya/Aza 5, Pred/Aza 3
85·7 (1·51)	39·5 (4·20)	24·6 (3·37)	2·62 (0·73)	134·8 (17·0)	Cya/Aza 8, Pred/Aza 0

s no significant difference between groups A and B for any of these variables.

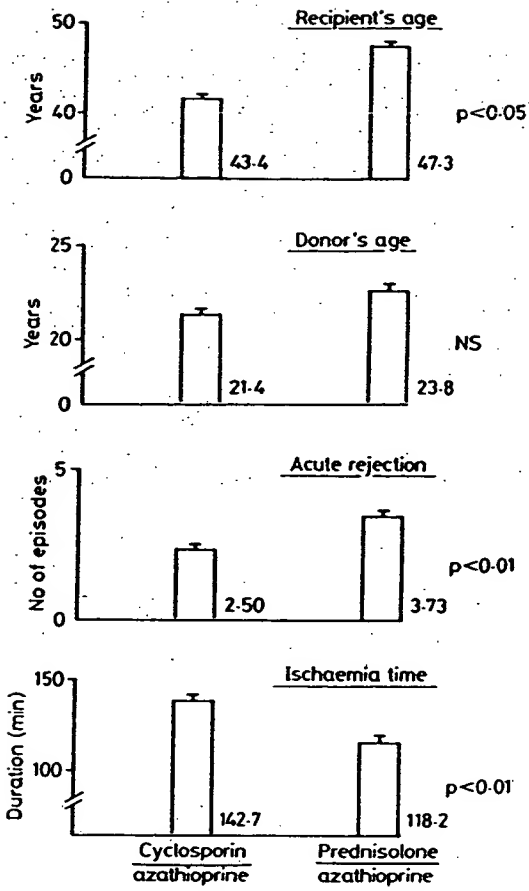


Fig 6 *Distribution of variables in groups treated with cyclosporin/azathioprine or prednisolone/azathioprine.*

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30. Abbreviations for the amino acid residues are A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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Isolation of the Cyclosporin-Sensitive T Cell Transcription Factor NFATp

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Nuclear factor of activated T cells (NFAT) is a transcription factor that regulates expression of the cytokine interleukin-2 (IL-2) in activated T cells. The DNA-binding specificity of NFAT is conferred by NFATp, a phosphoprotein that is a target for the immunosuppressive compounds cyclosporin A and FK506. Here, the purification of NFATp from murine T cells and the isolation of a complementary DNA clone encoding NFATp are reported. A truncated form of NFATp, expressed as a recombinant protein in bacteria, binds specifically to the NFAT site of the murine IL-2 promoter and forms a transcriptionally active complex with recombinant c-Fos and c-Jun. Antisera to tryptic peptides of the purified protein or to the recombinant protein fragment react with T cell NFATp. The molecular cloning of NFATp should allow detailed analysis of a T cell transcription factor that is central to initiation of the immune response.

Nuclear factor of activated T cells is an inducible DNA-binding protein that binds to two independent sites in the IL-2 promoter (*1*, *2*). NFAT is a multisubunit transcription factor (3) consisting of at least three DNA-binding polypeptides, the preexisting subunit NFATp (*4–6*) and homodimers or heterodimers of Fos and Jun family proteins (6–9). NFATp is present in the cytosolic fraction of unstimulated T cells (*3–7*); after T cell activation, it is found in nuclear extracts and forms DNA-protein complexes with Fos and Jun family members at the NFAT sites of the IL-2 promoter (*3*, *5–9*). NFATp has also been implicated in the transcriptional regulation of other cytokine genes, including the genes for granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-3, IL-4, and tumor necrosis factor–α (TNF-α) (*10*).

NFATp is the target of a Ca^{2+}-dependent signaling pathway initiated at the T cell receptor (*3*, *4*, *6*, *7*, *11–13*). The rise in intracellular free Ca^{2+} in activated T cells results in an increase in the phosphatase activity of the Ca^{2+}- and calmodulin-dependent phosphatase calcineurin (*14*). NFATp is a substrate for calcineurin in vitro (*4*, *6*) and is thought to be dephosphorylated by calcineurin in activated T cells, resulting in its translocation from the cytoplasm to the nucleus (*13*). Cyclosporin A (CsA) and FK506, which act as a complex with their respective intracellular receptors to inhibit the phosphatase activity of calcineurin (*15*), block the dephosphorylation of NFATp (*4*) and the appearance of NFAT in nuclear extracts of stimulated T cells (*2*, *3*, *7*, *12*). This mechanism may account for the sensitivity to cyclosporin of IL-2 and other cytokine genes (*10*, *13*) and thus for the profound immunosuppression caused by CsA and FK506 (*13*).

NFATp was purified from the C1.7W2 cell line (*16*), a derivative of the murine T cell clone Ar-5 (*17*), by ammonium sulfate fractionation followed by successive chromatography on a heparin-agarose column and an NFAT oligonucleotide affinity column (*18*). A silver-stained SDS gel of the purified protein showed a major broad band migrating with an apparent molecular size of ~120 kD (Fig. 1, top). We have shown that this band contains a DNA-binding phosphoprotein that is dephosphorylated by calcineurin to yield four sharp bands migrating with apparent molecular sizes of ~110 to 115 kD (6). NFATp DNA-binding activity was demonstrable in protein eluted from the SDS gel and renatured (*4*), and more than 90% of the activity recovered from the gel comigrated with the ~120-kD band (Fig. 1, lane 7). The faster migrating complexes formed with proteins of slightly smaller molecular size (lanes 8 to 11) most likely derive from partial proteolysis. The purified protein binds to the NFAT site with the appropriate specificity and forms a DNA-protein complex with recombinant Fos and Jun (6).

To confirm that the 120-kD protein was the preexisting subunit of the T cell transcription factor NFAT, we used antisera to tryptic peptides derived from the 120-kD protein (*18*). When one such antiserum (to peptide 72) was included in the binding reaction, it "supershifted" the NFATp-DNA complex formed by the cytosolic fraction from unstimulated T cells (Fig. 2, lane

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3), as well as both NFAT complexes formed by nuclear extracts from stimulated T cells (lane 8). The effect of the serum was prevented by preincubation with its cognate peptide (lanes 4 and 9), but not by preincubation with a different peptide (lanes 5 and 10). Preimmune serum had no effect on binding (lanes 2 and 7). Similar effects were seen with antisera to peptides 23.1 and 25. These data demonstrate that the purified protein is NFATp.

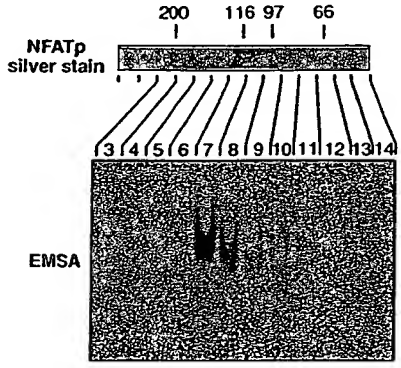

Fig. 1. Analysis of purified NFATp by renaturation of NFATp activity after SDS-PAGE. **(Top)** Purified NFATp (50 ng) was subjected to electrophoresis on an analytical 6% SDS-polyacrylamide gel and subsequently silver-stained (Pierce Gel-code kit). **(Bottom)** A second lane of the same gel was loaded with 50 ng of the purified protein. After electrophoresis, the gel was sliced, proteins were eluted from gel slices and renatured, and the fractionated proteins were evaluated in an electrophoretic mobility-shift assay for the ability to bind to the NFAT site of the murine IL-2 promoter (*4*).

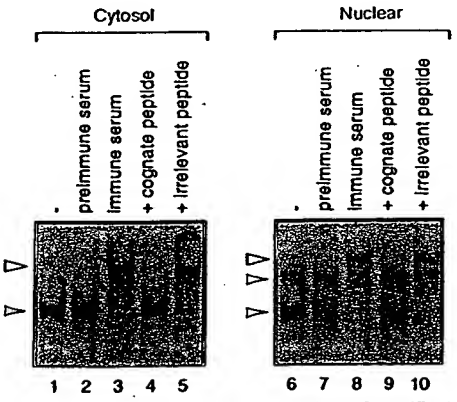

Fig. 2. Antisera to tryptic peptides of purified NFATp react with NFATp in T cell extracts. Cytosolic extracts (*4*) from unstimulated Ar-5 T cells (lanes 1 to 5) or nuclear extracts (*23*) from Ar-5 T cells stimulated with antibody to CD3 (anti-CD3) (lanes 6 to 10) were incubated without antiserum (lanes 1 and 6), with antiserum to peptide 72 (immune, lanes 3 and 8), or with serum from the same rabbit taken before immunization (preimmune, lanes 2 and 7), then analyzed by gel-shift assay for binding to the NFAT oligonucleotide (*7*). For peptide competition, 1 μg of peptide 72 (lanes 4 and 9) or peptide 25 (lanes 5 and 10) was mixed with the antiserum before it was added to cell extracts. Filled arrowheads identify the cytosolic NFATp, nuclear NFATp, and nuclear NFATp-Fos-Jun complexes; open arrowheads indicate the "supershifted" complexes; the unmarked complex results from binding of serum proteins.

In order to isolate a cDNA clone for NFATp, we used degenerate oligonucleotides based on the sequences of two tryptic peptides of purified NFATp in a polymerase chain reaction (PCR) to amplify an ~800-bp fragment from C1.7W2 cDNA, and the fragment was used to screen a cDNA library from murine T cells (*19*). The longest clone isolated contains an insert of ~4.5 kb in length, with an open reading frame extending 2672 base pairs (bp) from the 5′ end of the insert and with ~1.8 kb of 3′ untranslated region that does not extend to the polyadenylate tail. The open reading frame encodes a polypeptide of 890 amino acids

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1
GSSASFISDTFSPYTSPCVSPWNAGPDDLCPQFQNIPAEYSPRTSPIMSP

51
RTSLAEDSCLGRHSPVPRPASRSSSPGAKRRHSCAEALVAPLPAASPQRS

101
RSPSPQPSPHVAPQDDSIPAGYPPTAGSAVLMDALNTLATDSPCGIPSKI

151
WRTSPDPTPVSTAPSKAGLARHIYPTVEFLGPCEQEERRNSAPESILLVP
   XXXX----23.3-------|
201                   ▽
PTWPEQLVPAIPICSIPVTASLPPLEWPLSNQSGSYELRIEVQPKPHHRA
    |-----------72--------x-----|
251
EYETEGSRGAVKAPTGGHPVVQLHGYMENKPLGLQIFIGTADERILKPHA

301
FYQVHRITGKTVTTTSYEKIVGNTKVLEIPLEPKNNMRATIDCAGILKLR
            |--10.2--|
351
NADIELRKGETDIGRKNTRVRLVFRVHVPEPSGRIVSLQAASNPIECSQR

401
SAHELPMVERQDMDSCLVYGGQQMILTGQNFTAESKVVFMEKITDGQQIW
|-----------48----------|              |-23.2-|
451
EMEATVDKDKSQPNMLFVEIPEYRNKHIRVPVKVNFYVINGKRKRSQPQH

501
FTYHPVPAIKTEPSDEYEPSLICSPAHGGLGSQPYYPQHPMLAESPSCLV

551
ATMAPCQQFRSGLSSPDARYQQQSPAAALYQRSKSLSPGLLGYQQPSLLA
            |-10.1--|----23.1-----|
601
APLGLADAHRSVLVHAGSQGQGQGSTLRHTSSASQQASPVIHYSPINQQL

651                                   ▽
RGGGHQEFQHIMYCENFGPSSARPGPPPINQCQRLSPGAIPTVIQQQTAP
                                      |------25-------
701
SQRAAKNGPSDQKEALPTGVTVKQEQNLDQTYLDDAATSESWVGTERYIE
---|
751
KKFWKKTLVQPGLLPSFLLLGSLSAGPRSQTPSERKPIEEDVPLSCSQIA

801
WCCQHPLGTCPVLPGPLAVEWWEGQLGRGLEPIPWAPDSAGSLHEVDSVG

851
LAGVVGHVLLTLMHHFSMDQNQTPSPEWQRHKEVASPGWI
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Fig. 3. Deduced amino acid sequence of NFATp. The underlined sequences match the sequences of tryptic peptides from purified NFATp. X in the underlining for peptides 23.3 and 72 indicates positions at which the identity of the amino acid could not be determined unambiguously. The arrowheads delimit the NFATp sequence contained within the recombinant protein that was expressed in bacteria. The sequence of murine NFATp has been deposited with GenBank (accession number U02079). Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

(Fig. 3) that contains eight of nine tryptic peptides identified by sequencing of purified NFATp. The cDNA insert may lack a small amount of coding sequence at the 5′ end, because the predicted molecular size of the encoded protein (97 kD) is somewhat smaller than the apparent molecular size of dephosphorylated NFATp [110 to 115 kD (*6*)] and because one tryptic peptide from purified NFATp is unaccounted for in the encoded protein. A search of the Genbank DNA and protein databases with the Blast algorithm (*20*) indicated that the cDNA encodes a previously unidentified protein. A 464–amino acid fragment containing the DNA-binding domain displayed a limited similarity to the rel homology domain of human and murine RelA (p65) (18.9 and 17.8% amino acid identity, respectively, over 428 amino acids). A preliminary analysis of additional cDNA clones indicates that T cells express at least three forms of NFATp related to each other by alternative splicing and differing at their COOH-termini.

The T cell lines C1.7W2 and Ar-5, but not L cells, express NFATp mRNA (Fig. 4), consistent with our previous demonstration that NFATp protein is present in T cells but not in L cells (*4*). The ~800-bp PCR fragment hybridized to a transcript of ~8–9 kb expressed in the C1.7W2 T cell line used for purification of NFATp (lane 1) and in the untransformed T cell clone Ar-5 used to generate the cDNA library (lane 2), but did not hybridize to any transcript expressed in L cells (lane 3). Two other cDNA probes representing different parts of the coding region of NFATp gave similar results. We are presently undertaking a

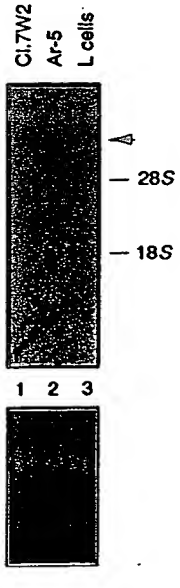

Fig. 4. Northern (RNA) analysis of NFATp mRNA in T cell and fibroblast cell lines. Cytoplasmic RNA from the murine T cell clone Ar-5, the transformed T cell line C1.7W2, and the murine fibroblast L cell line were separated by electrophoresis in formaldehyde gels, transferred to nylon, and hybridized with a labeled fragment of NFATp coding sequence corresponding to the ~800-bp PCR product. The positions of the major NFATp transcript (arrow) and of the 28*S* and 18*S* ribosomal RNAs are indicated. The lower panel shows ethidium bromide staining of the RNA before transfer to nitrocellulose indicating that the RNA was intact and that equivalent amounts of RNA were loaded in each lane.

systematic analysis of the tissue distribution of NFATp by protein immunoblotting analysis and quantitative PCR.

To test directly whether the cDNA encoded a protein with the characteristics of NFATp, we examined the ability of a recombinant fragment of the protein to bind to the NFAT site of the murine IL-2 promoter and to associate with Fos and Jun. A 464–amino acid fragment of the protein (sequence between arrowheads in Fig. 3) was expressed as a hexahistidine-tagged protein in bacteria (*21*). This recombinant protein bound to the NFAT oligonucleotide in a gel-shift assay (Fig. 5A, lane 1). Its binding specificity was identical to that of authentic T cell NFATp (*4–7*), as judged by competition with excess unlabeled NFAT oligonucleotide (lane 2) and the mutant NFAT oligonucleotides M1 to M3 (lanes 3 to 5). The M1 oligonucleotide (lane 3) is mutated in four bases remote from the NFAT binding site and competes as strongly for binding as the authentic NFAT oligonucleotide; the M2 oligonucleotide (lane 4) is mutated in four bases located between the M1 and M3 regions and competes with intermediate efficiency; and the M3 oligonucleotide (lane 5) is mutated in the GGAA tetranucleotide sequence essential for binding of NFATp (*4–7, 22, 23*) and does not compete for binding. Methylation interference analysis also showed that binding of the recombinant protein to the NFAT site required the GGAA core binding region, as previously demonstrated for NFAT (*22, 23*). Like NFATp purified from T cells (*6*), the recombinant protein associated with homodimers of c-Jun or with heterodimers of c-Fos and c-Jun, but not with c-Fos alone, to form a DNA-protein complex that migrated with slower mobility than the NFATp-DNA complex in a gel-shift assay (lanes 7 to 9). The c-Fos and c-Jun proteins do not bind to the NFAT oligonucleotide in the absence of NFATp (lane 10). The complex containing c-Fos and c-Jun resembled the nuclear complex of NFATp, Fos, and Jun in that its formation was competed by excess unlabeled activator protein 1 (AP-1) oligonucleotide. These data indicate that a ~50-kD fragment of NFATp is sufficient to account for the DNA-binding properties of NFATp and for its ability to associate with Fos and Jun proteins.

Evidence that the cDNA clone encodes NFATp was provided by the ability of antisera to the recombinant protein to react specifically with NFATp from cytosolic or nuclear extracts of T cells. When serum from a rabbit immunized with the recombinant protein (*21*) was included in the gel-shift assay, a small proportion of the NFATp-DNA complexes were "supershifted" (Fig. 6, lane 3), and most of the

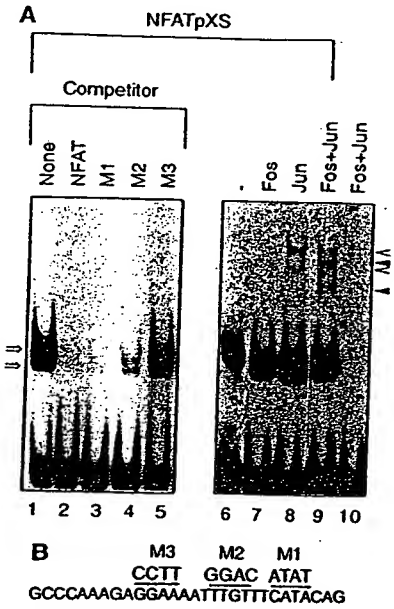

Fig. 5. Binding of a recombinant fragment of NFATp (NFATpXS) to DNA and association with Fos and Jun proteins. (**A**) Lanes 1 to 5, binding of the recombinant fragment of NFATp (*21*) to the distal NFAT site of the murine IL-2 promoter was assessed by electrophoretic mobility-shift assay (*4*) in the presence or absence of a 200-fold excess of unlabeled competitor oligonucleotides. The arrows indicate two DNA-protein complexes formed with NFATpXS. Lanes 7 to 9, full-length recombinant c-Fos and c-Jun proteins (*30*) were included in the binding reactions. The open arrows indicate Jun-Jun-NFATpXS complexes, whereas the closed arrows indicate Fos-Jun-NFATpXS complexes. Lane 10, Fos and Jun proteins do not bind to the NFAT oligonucleotide. (**B**) Sequences of competitor oligonucleotides.

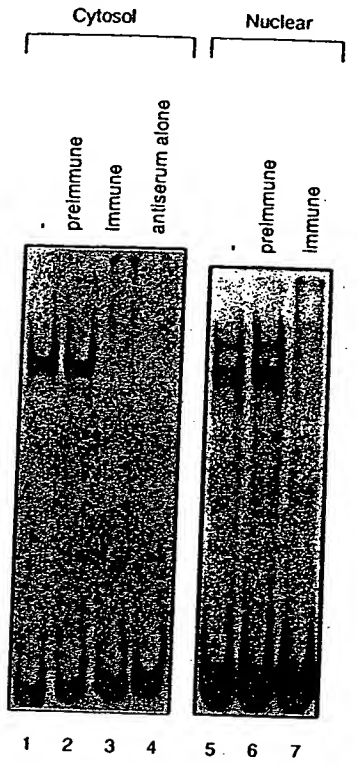

Fig. 6. Antisera to recombinant NFATp react with NFATp in T cell extracts. Cytosolic extracts from unstimulated Ar-5 T cells or nuclear extracts from cells stimulated with anti-CD3 were incubated without antiserum (lanes 1 and 5), with an antiserum to the recombinant NFATp fragment (*21*) (lanes 3 and 7), or with preimmune serum from the same rabbit (lanes 2 and 6), followed by gel-shift analysis of binding to the NFAT oligonucleotide.

DNA-protein complexes appeared to be in large aggregates (lanes 3 and 7). The predominance of large aggregates probably reflects recognition by the serum of multiple antigenic determinants on NFATp. Preimmune serum from the same rabbit did not alter the mobility of NFATp-DNA and NFAT-DNA complexes (lanes 2 and 6).

To examine the role of the cloned NFATp protein in transcription, we tested the effect of the recombinant NFATp fragment on transcription in vitro from a template containing three NFAT sites upstream of the murine IL-2 promoter (Fig. 7). The same plasmid has been used to demonstrate transcriptional activation in vivo in response to stimulation with antigen (*7*). A combination of the recombinant NFATp fragment with c-Fos and c-Jun, or with c-Jun only, activated transcription from this construct (Fig. 7, lanes 2 and 3). In combination with NFATp, a Jun deletion derivative (J91-334) lacking the NH_2-terminal repressor domain was a more potent activator than full-length Jun (lanes 6, 7, and 14), as previously observed for transcriptional activation by Jun at AP-1 sites (*24*). In contrast, neither the truncated NFATp alone nor AP-1 proteins alone had a notable effect (lanes 5, 10, and 11). Truncated Fos and Jun proteins (F139-200 and J241-334) containing the dimerization and DNA-binding domains but lacking transcriptional activation domains are able to form a complex with NFATp (*6*). However, they did not activate transcription in conjunction with truncated NFATp (lanes 8 and 9), indicating that the truncated NFATp is not transcriptionally active in the absence of Fos and Jun. No notable transcriptional activation was observed when a template containing a mutated NFAT site incapable of binding NFATp was used (lanes 15 to 17). Moreover, the truncated NFATp had no effect on transcription activated by Fos and Jun on a template containing an AP-1 site (lanes 18 to 24), consistent with our previous observation that NFATp does not form a complex with Fos and Jun on the AP-1 site (*6*).

These data show that truncated NFATp forms a transcriptionally active complex with Fos and Jun at the IL-2 promoter

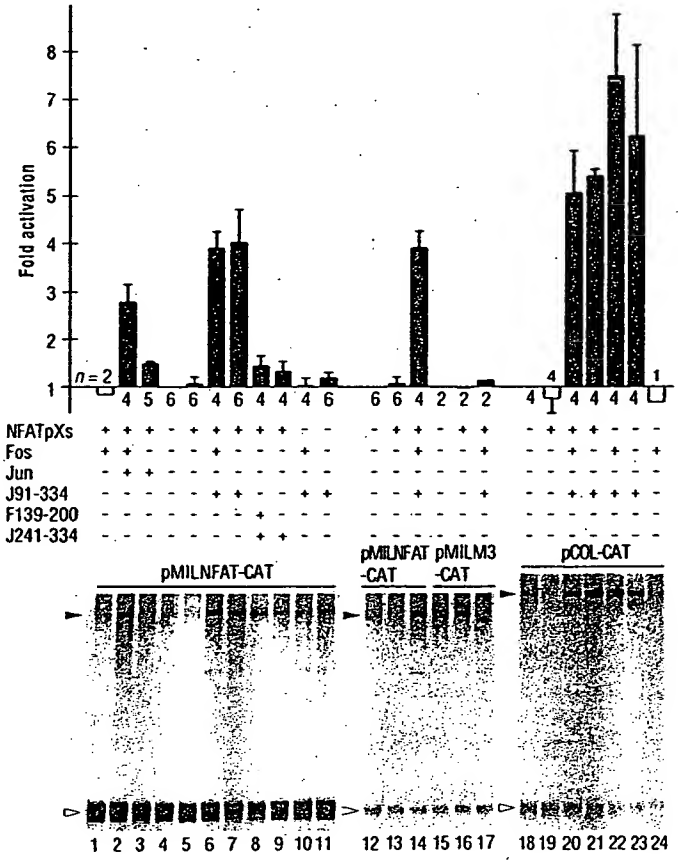

Fig. 7. Transcriptional activation by NFATp, c-Fos, and c-Jun on different templates. The templates indicated above the lanes were incubated with the proteins listed and transcribed in vitro in Namalwa nuclear extracts (*24, 7*). The pMILNFAT-CAT construct (lanes 1 to 14) contains three NFAT sites upstream of the basal IL-2 promoter (*7*). The pMILM3-CAT construct (lanes 15 to 17) contains four NFAT sites in which critical contact residues have been altered (*7*). The pCOL-CAT construct contains residues −73 to +63 of the human collagenase promoter including an AP-1 site (*24*). Transcripts were resolved on denaturing polyacrylamide gels and quantitated with the use of a phosphorimager. The filled arrowheads point to the specific transcripts and the open arrowheads to internal controls. Transcription in the presence of different combinations of proteins was expressed relative to a reaction in the absence of recombinant proteins (fold activation). The average of several independent experiments (number shown at the base of each bar) and the standard deviation in cases where more than three independent experiments were performed are shown (solid bars, activation; open bars, repression).

NFAT site. They are consistent with the interpretation that NFATp primarily determines the DNA-binding specificity of the NFAT complex in vivo, whereas at least a portion of the transcriptional activity is provided by Fos and Jun. Because the current experiments were performed with a truncated NFATp, they do not exclude the possibility that full-length NFATp has a transcriptional activation domain that can function in the absence of Fos and Jun. However, there is evidence suggesting that Fos and Jun family proteins are required along with NFATp to activate transcription at the IL-2 promoter NFAT site in vivo, since mutations in the NFAT site that prevent the association of Fos and Jun with NFATp abolish the function of this site in activated T cells (*8*).

The cDNA clone reported here fulfills four essential criteria defining NFATp: (i) the mRNA is expressed in T cells but not in fibroblasts, (ii) a recombinant fragment of the protein binds specifically to the NFAT site, (iii) the recombinant protein fragment forms a transcriptionally active complex with Fos and Jun on the NFAT DNA sequence, and (iv) antibodies to the recombinant protein specifically react with NFATp in T cell extracts. The recombinant protein defines a functional 464–amino acid fragment of NFATp that contains the domains required for DNA binding and for formation of a transcriptionally active complex with Fos and Jun. The cloning of this previously uncharacterized DNA-binding protein makes possible detailed studies of its structure, its interactions with other transcription factors and with specific sites in DNA, its role in the induction of IL-2 and other cytokine genes, and its regulation by calcineurin during T cell activation.

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18. CI.7W2 cell extracts were prepared by NP-40 lysis and ammonium sulfate precipitation as described (*4*). The precipitated protein (1.2 g from 10^{11} cells) was dialyzed against buffer A [150 mM NaCl, 20 mM Hepes (pH 7.4), 2 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol], supplemented with protease inhibitors [aprotinin (100 μg/ml), 2.5 μM leupeptin, and 2 mM phenylmethylsulfonyl fluoride] and loaded onto a 30-ml heparin-agarose column (Sigma). The column was washed with 10 column volumes of the same buffer containing 200 mM NaCl, and bound protein was eluted with a linear gradient of 0.2 to 1.0 M NaCl in a total volume of 250 ml. The NFATp activity was determined by electrophoretic mobility-shift assay as described (*4, 7*). Active fractions were combined and dialyzed overnight against buffer A. The dialyzed pool (90 ml, 95 mg of protein) was loaded in 20-mg batches onto a 1-ml high-capacity oligonucleotide affinity column (*25*) in the presence of sheared herring sperm DNA (200 μg/ml). The column was washed with the same buffer, and NFAT was eluted with a linear gradient of 0.15 to 1.0 M NaCl. The NFAT activity eluted in fractions between 0.4 and 0.6 M NaCl. The peak fractions from several separate fractionations were combined, dialyzed against buffer containing 150 mM NaCl, and reloaded onto the same affinity column. After two cycles over the affinity column, ~10 μg of highly purified NFATp was obtained. This material bound specifically to the NFAT site, was a phosphoprotein substrate for calcineurin, and associated with c-Fos and c-Jun to form the NFAT-Fos-Jun ternary complex on the NFAT site oligonucleotide (*6*). Renaturation from gel slices and electrophoretic mobility-shift assays were done as described (*4*). The purified NFATp protein was acetone precipitated, subjected to electrophoresis on a 6% SDS-polyacrylamide gel, and transferred to nitrocellulose. The NFATp band was localized by Ponceau Red staining, excised, and digested with trypsin in situ. The resultant peptides were separated by microbore high-performance liquid chromatography, analyzed by laser desorption mass spectroscopy, and Edman microsequenced. Strategies for the selection of peptide fractions and their microsequencing have been described (*26*). For generation of antisera to peptide 72, rabbits were immunized with a 21–amino acid synthetic peptide conjugated to keyhole limpet hemocyanin (*27*).
19. Degenerate oligonucleotides based on the sequences of peptides 23.2 and 25 were used in a PCR to amplify an ~800-bp fragment from CI.7W2 cDNA. The fragment was used to screen an amplified cDNA library (representing 10^6 primary plaques) in λ ZAPII (Stratagene), generated by oligo(dT) and random priming of cytoplasmic polyadenylated mRNA from Ar-5 T cells. After plaque purification of the recombinant λ bacteriophage clones and excision of pBluescript phagemids carrying the cDNA inserts, the coding sequences of several cDNA clones were determined by sequencing of both strands using the dideoxy chain termination method (*28*).
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21. A cDNA fragment common to all the alternatively spliced cDNAs was excised by digestion with Xho I and Sma I, subcloned into the vector pQE-31 (Qiagen), and expressed as a hexahistidine-tagged protein in bacteria (*29*). The expressed protein contained an additional 18 vector–encoded amino acids (MRGSHHHHHHTAPHASSV) at the NH_2-terminus and 9 amino acids (VDLEPSLIS) at the COOH-terminus of the sequence indicated between the arrowheads in Fig. 3. The recombinant protein was purified by chromatography on a nickel-chelate column in 8 M urea, and elution was with 250 mM imidazole. After dialysis against buffer A (*18*), the protein was assayed for DNA binding and association with Fos and Jun as described for NFATp purified from T cells (*6*). To generate antisera, we immunized rabbits with the purified recombinant NFATp.
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31. In vitro transcription reactions were carried out in Namalwa nuclear extracts as described (*24*). Fos and Jun proteins and truncated NFATp (NFATpXS) were used at 500 μM, and plasmid templates linearized by Eco RI were used at 80 μg/ml. Transcripts purified from the reactions were analyzed by polyacrylamide gel electrophoresis (PAGE) and quantitated with the use of a phosphorimager.
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Nonuniform Probability of Glutamate Release at a Hippocampal Synapse

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A change in the probability of neurotransmitter release (P_r) is an important mechanism underlying synaptic plasticity. Although P_r is often assumed to be the same for all terminals at a single synapse, this assumption is difficult to reconcile with the nonuniform size and structure of synaptic terminals in the central nervous system. Release probability was measured at excitatory synapses on cultured hippocampal neurons by analysis of the progressive block of *N*-methyl-D-aspartate receptor–mediated synaptic currents by the irreversible open channel blocker MK-801. Release probability was nonuniform (range of 0.09 to 0.54) for terminals arising from a single axon, the majority of which had a low P_r. However, terminals with high P_r are more likely to be affected by the activity-dependent modulation that occurs in long-term potentiation.

The probability of transmitter release (P_r) rom individual synaptic terminals can be stimated from excitatory postsynaptic cur-ent (EPSC) amplitude fluctuations by the se of a statistical model of the release rocess (quantal analysis) (*1*, *2*). This ap-roach is complicated if P_r is not the same or all terminals (*2*). It is difficult to esti-ate P_r at central synapses because minia-ure EPSC amplitudes are close to the trinsic recording noise level and are highly variable (*2*, *3*). Furthermore, the assumptions underlying statistical models of transmitter release may not always be appropriate at central synapses (*2*, *4*). For instance, the standard binomial model assumes that P_r is uniform at all synaptic terminals. To test directly for nonuniform P_r, we developed an alternative to quantal analysis.

Whole-cell recordings were made from single cultured rat hippocampal neurons that formed recurrent (autaptic) synapses (*5*). Recordings of *N*-methyl-D-aspartate (NMDA) receptor–mediated EPSCs were made before and during exposure to the NMDA open channel blocker, MK-801 (5 to 20 μM) (*6*). Channels were irreversibly blocked under our recording conditions (*7*). The MK-801 exposure increased the decay rate of the EPSC (Fig. 1, A and B) and, with repeated stimuli, progressively reduced its amplitude (Fig. 1A). The progressive block rate was measured by the fitting of a single exponential to the EPSC peak amplitude plotted against stimulation number. The rate of progressive block reflects, in part, P_r. If P_r is high then more terminals will release transmitter, more postsynaptic NMDA channels will open, and the progressive block should be more rapid. Consistent with this hypothesis, the progressive block rate was proportional to P_r (Fig. 1, C and D). Raising the calcium concentration increased the EPSC amplitude in the absence of MK-801 (Fig. 1C), and the progressive block rate in MK-801 (5 μM) increased proportionally (Fig. 1D) (*8*). Thus, the progressive block rate provides a relative measure of P_r. To obtain a quantitative measure of P_r, estimates of the time course of glutamate in the synaptic cleft, NMDA channel open probability (P_o) and MK-801 binding rate are also required. All these parameters have been measured (*6*, *9*–*11*), but P_o was obtained from outside-out patch or whole-cell recordings that include extrasynaptic channels (*6*, *11*). Therefore, we examined the P_o of synaptically activated NMDA channels.

Channel open probability has been calculated from the progressive block of NMDA currents by MK-801 (*11*), but this approach cannot be applied to synaptic currents because progressive block is also influenced by P_r. However, the faster decay rate of NMDA receptor–mediated EPSCs in the presence of MK-801 (Fig. 1B) can be used to estimate P_o. This rate can be used because the irreversible block of open channels early in the synaptic response prevents reopenings later in the response and thus accelerates the EPSC decay (*11*). This acceleration increases with increasing P_o. A chemical kinetic model (*9*) (Fig. 2A inset) was used to fit the time course of the synaptic current recorded in the absence and presence of MK-801 (5 μM) (Fig. 2A) (*12*). The channel opening rate was the only free parameter in the kinetic model that affected the change in decay rate produced by MK-801, and it had an optimum value of $12.4 \pm 0.7\ s^{-1}$ (mean ± SEM, $n = 11$). Channel open probability was then calculated from the opening and closing rates (τ_o and τ_c, respectively) with the equation $P_o = \tau_o/(\tau_o + \tau_c)$; P_o was 0.053 ± 0.003 ($n = 11$). The open probability of an NMDA channel at the peak of a synaptic response (P_o*) was also calculated from the optimum kinetic model to be 0.041 ± 0.003 ($n = 11$). This probability is less than P_o because some desensitization and agonist dissociation occur during the rising phase of the response. Our estimate of P_o* was significantly lower than that for channels in outside-out patches (P_o* = 0.27) (*11*). This discrepancy was not due to differences in the analysis procedures (*13*). The lower

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Blockade of T-Cell Activation by Dithiocarbamates Involves Novel Mechanisms of Inhibition of Nuclear Factor of Activated T Cells

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Dithiocarbamates (DTCs) have recently been reported as powerful inhibitors of NF-κB activation in a number of cell types. Given the role of this transcription factor in the regulation of gene expression in the inflammatory response, NF-κB inhibitors have been suggested as potential therapeutic drugs for inflammatory diseases. We show here that DTCs inhibited both interleukin 2 (IL-2) synthesis and membrane expression of antigens which are induced during T-cell activation. This inhibition, which occurred with a parallel activation of c-Jun transactivating functions and expression, was reflected by transfection experiments at the IL-2 promoter level, and involved not only the inhibition of NF-κB-driven reporter activation but also that of nuclear factor of activated T cells (NFAT). Accordingly, electrophoretic mobility shift assays (EMSAs) indicated that pyrrolidine DTC (PDTC) prevented NF-κB, and NFAT DNA-binding activity in T cells stimulated with either phorbol myristate acetate plus ionophore or antibodies against the CD3–T-cell receptor complex and simultaneously activated the binding of AP-1. Furthermore, PDTC differentially targeted both NFATp and NFATc family members, inhibiting the transactivation functions of NFATp and mRNA induction of NFATc. Strikingly, Western blotting and immunocytochemical experiments indicated that PDTC promoted a transient and rapid shuttling of NFATp and NFATc, leading to their accelerated export from the nucleus of activated T cells. We propose that the activation of an NFAT kinase by PDTC could be responsible for the rapid shuttling of the NFAT, therefore transiently converting the sustained transactivation of this transcription factor that occurs during lymphocyte activation, and show that c-Jun NH_2-terminal kinase (JNK) can act by directly phosphorylating NFATp. In addition, the combined inhibitory effects on NFAT and NF-κB support a potential use of DTCs as immunosuppressants.

The interaction of T lymphocytes with antigens triggers a complex signaling cascade that switches on the gene program leading to T-cell activation. During this process, T cells express the autocrine growth factor interleukin 2 (IL-2), which promotes cell proliferation by interacting with its receptor, also expressed by activated T cells. The transcriptional regulation of the IL-2 gene has been extensively analyzed with the IL-2 promoter, a 275-bp region located upstream of the transcriptional start site of the gene. *Cis*-Acting elements for several transcription factors have been identified within this regulatory region. The factors which bind to these motifs include AP-1, NF-κB, Oct-1, and nuclear factor of activated T cells (NFAT) family proteins (reviewed in references 15 and 26).

The transcription factor NFAT plays an essential role in IL-2 gene expression (59). Binding sites for NFAT have also been found within the promoter regions of several cytokines, including granulocyte-macrophage colony-stimulating factor, tumor necrosis factor alpha, IL-3, IL-4, and IL-5 (10–12, 17, 27, 34, 44, 64). NFAT is composed of a complex whose binding specificity is mediated by a cytosolic subunit and an inducible nuclear component comprised of AP-1 family members. The cytoplasmic subunit is encoded by a family of genes constituted by at least four structurally related NFAT members, NFATp/NFAT1, NFATc, NFAT3, and NFATX/NFAT4/NFATc3 (20, 23, 31, 35, 37, 43). These members translocate to the nucleus upon calcium mobilization during T-cell activation involving the calcineurin-dependent dephosphorylation of the transcription factor. Blockade of this pathway by the immunosuppressive drugs cyclosporin A (CsA) and FK 506 results in inhibition of the phosphatase activity of calcineurin, thus preventing the subsequent dephosphorylation and translocation of NFAT to the nucleus. Hence, NFAT can be considered a secondary target of the action of the immunosuppressive drugs, whose inhibition accounts, at least in part, for the transcriptional inhibitory effects of the immunosuppressants (28, 55). In the nucleus, NFAT family proteins can interact with the inducible nuclear component formed by Fos and Jun family members to bind cooperatively and transactivate NFAT sites in vitro (8, 15, 25, 26, 48).

Dithiocarbamates (DTCs) are antioxidant compounds that have been shown to exert opposite effects on the activity of NF-κB and AP-1 transcription factors. In T cells, DTCs inhibit activation of NF-κB by a number of stimuli that promote the production of reactive oxygen intermediates proposed as common second messengers in the activation of the transcription factor (3, 52, 57). In contrast, AP-1 has been shown to be activated by DTCs in a number of cell types, including T lymphocytes (18, 57, 58). Although the precise primary cellular targets involved in DTC-mediated changes in the activity of these transcription factors have not been identified so far, the inhibition by pyrrolidine DTC (PDTC) of NF-κB involves the

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blockade of IκB-α phosphorylation (66), whereas PDTC-mediated AP-1 activation in T lymphocytes is accompanied by a strong and sustained activation of c-Jun NH_2-terminal kinase (JNK) (18).

Since DTCs affect the activity of transcription factors important for gene regulation during the T-cell activation process, we analyzed here the effects of these agents on IL-2 gene expression and on the activity of its promoter region, which is in turn regulated by multiple transcription factors that integrate signals transmitted from different pathways. We found that despite the activation exerted on AP-1, DTCs inhibited NFAT-dependent transactivation and binding by mechanisms different from those mediated by the immunosuppressive drugs FK 506 and CsA. We discuss the potential benefits derived from the use of DTCs as immunosuppressants and speculate on the putative involvement of an NFAT kinase mediating the inhibitory effects of these agents on the NFATp transactivation functions.

MATERIALS AND METHODS

Cell culture and reagents. Jurkat and PEER cells (αβ and γδ T-cell lines) were maintained in RPMI 1640 medium (Gibco-BRL) supplemented with 10% fetal bovine serum. Peripheral blood mononuclear cells were isolated from healthy volunteers, and venous blood was drawn with heparin syringes and diluted in an equal volume of saline solution. The suspension was layered over a Ficoll-Hypaque cushion and centrifuged. Mononuclear cells were collected, washed, and resuspended in RPMI 1640 medium supplemented with 10% fetal bovine serum. The cells were then incubated at 37°C for 1 h on plastic dishes to remove monocytes. The peripheral blood lymphocytes (PBLs) (5×10^5 per well) were then stimulated with immobilized anti-CD3 monoclonal antibody (MAb) in a 24-well tissue culture plate previously precoated for 2 h with 100 μl of a 10-μg/ml solution of UCHT.1 anti-CD3 antibody (9) at 37°C. Phorbol 12-myristate 13-acetate (PMA), PDTC, diethyl DTC (DDTC), disulfiram, butylated hydroxyanisole (BHA), *N*-acetylcysteine (NAC), and the calcium ionophore A23187 were purchased from Sigma. CsA was obtained from Sandoz.

Antibodies, flow cytometry analysis, and IL-2 immunoassay. After different treatments, cells were collected by centrifugation, resuspended in phosphate-buffered saline (PBS), and incubated at 4°C for 30 min with hybridoma culture supernatant of TP1/55 anti-CD69 MAb (50) or fluorescein isothiocyanate-conjugated anti-CD25 antibody from Becton Dickinson. Cells were washed with PBS and resuspended in 300 μl of propidium iodide (2 ng/μl), and bound anti-CD69 antibody was detected with fluorescein isothiocyanate-conjugated rabbit $F(ab')_2$ anti-mouse immunoglobulin G (IgG) (Dako A/S, Roskilde, Denmark). The supernatant from the myeloma P3X63 was used as a negative control. The anti-CD3 MAbs were purified by affinity chromatography on a protein A-Sepharose column. When cells were activated with anti-CD3 antibodies, the biotinylated TP1/55 MAb was used to monitor CD69 expression. In this case, biotinylated TEA 2/1 (anti-ELAM-1 MAb) was used as a negative control. Samples were analyzed by flow cytometry in a FACScan cytofluorometer (Becton Dickinson, Mountain View, Calif.). Analysis was performed with viable cells (typically higher than 95%) as determined by staining with the fluorochrome propidium iodide. Since we observed variability among different batches of Jurkat cells regarding the doses of DTCs required to achieve maximal inhibition of membrane markers of T-cell activation, we routinely performed flow cytometry analysis of CD69 with doses of PDTC ranging between 50 and 200 μM to select the minimal dose of PDTC that achieved the maximal inhibition in activated Jurkat cells.

IL-2 levels were measured with an enzyme-linked immunosorbent assay kit purchased from Amersham. Cell supernatants from Jurkat T cells were collected 20 h after stimulation.

Plasmid constructs. The reporter constructs NFAT-Luc, containing three tandem copies of the NFAT binding site fused to the IL-2 minimal promoter, and IL-2-Luc, containing the region spanning from −326 to +45 of the human IL-2 promoter or enhancer have been described previously (16) and were provided by G. Crabtree. The pKBF-Luc construct includes a trimer of the NF-κB motif of the *H-2K*^{b} gene upstream of the herpes simplex virus thymidine kinase minimal promoter driving the luciferase reporter gene (73).

The GAL4 plasmids RSV-GAL4-DBD (RSV is Rous sarcoma virus) and GAL4-hNFAT1(1–415) have been described previously (32). The GAL4-hNFAT1(1–415) plasmid encodes the transactivation domain of NFAT1/p (amino acids 1 to 415) in frame with the GAL4 DNA binding domain (DBD). RSV-GAL4-DBD is the parental vector including just the DBD (amino acids 1 to 147) of GAL4. RSV–GAL4–c-Jun (wild type) and RSV–GAL4–c-Jun $S_1 + S_2$, constructs that encode the wild-type transactivation domain of c-Jun and this domain mutated in its phosphorylation sites (Ser 63 and Ser 73), respectively (45), were gifts from P. Angel. The GAL4-Luc reporter plasmid, including five GAL4 DNA binding sites fused to the luciferase gene (provided by R. Perona) has been described previously (39).

Transient transfections and luciferase assays. Jurkat cells (2×10^7) were transiently transfected with 5 μg of luciferase reporter plasmid with 10 μg of Lipofectin reagent (Gibco BRL) for 8 h in 1 ml of OPTIMEM (Gibco BRL) or were cotransfected with 2 μg of GAL4 expression plasmids and 4 μg of GAL4-Luc construct, with 10 μg of Lipofectin reagent added for 8 h in 1 ml of OPTIMEM in transactivation experiments. The cells were then diluted in complete medium and after 36 h were preincubated or not with different concentrations of PDTC for 2 h and stimulated with PMA plus A23187 for 3 or 8 h as indicated. Cells were then collected by centrifugation and lysed according to the instructions of a Promega luciferase assay kit. The luciferase activity was measured for 30 s with a Lumat LB9501 luminometer (Berthold, Germany).

Nuclear extracts and EMSAs. For nuclear extracts, Jurkat cells were pretreated or not with 100 μM PDTC for 2 h and further stimulated for 1, 4, or 8 additional h with 20 ng of PMA per ml plus 1 μM A23187. PBLs were also pretreated or not with PDTC for 2 h and then washed with PBS and incubated with hybridoma culture supernatant of anti-CD3 SPV-T3 MAb (62) for 30 min at 4°C. Next, cells were incubated with Dynabeads M-450 coupled to sheep anti-mouse IgG (Dynal A.S., Oslo, Norway) for 30 min under bidirectional rotation at 4°C. The Dynabeads with T cells attached were collected with a suitable magnet, resuspended in ice-cold RPMI 1640 with 10% fetal bovine serum at 4°C, and incubated at 37°C for 15, 60, and 120 min in the presence or absence of PDTC. Small-scale nuclear extracts were then prepared according to a procedure described elsewhere (54) with some modifications. Briefly, cells were collected by centrifugation, washed once with PBS, and resuspended in 400 μl of ice-cold buffer A (10 mM HEPES [pH 7.6], 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.75 mM spermidine, 0.15 mM spermine, 1 mM dithiothreitol [DTT], 0.5 mM phenylmethylsulfonyl fluoride [PMSF], 10 mM Na_2MoO_4, 1 μg of pepstatin per ml, 2 μg each of leupeptin and aprotinin per ml). After 15 min on ice, 0.6% (vol/vol) Nonidet P-40 was added, and cells were vortexed and centrifuged in a microcentrifuge for 30 s at 15,000 × *g*. The nuclear pellet was extracted with 50 μl of buffer C (20 mM HEPES [pH 7.6], 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 10 mM Na_2MoO_4, 1 μg of pepstatin per ml, 4 μg of leupeptin per ml, 4 μg of aprotinin per ml) for 30 min on a rocking platform and further centrifuged at 15,000 × *g* for 10 min. The supernatants were collected and stored at −80°C. All steps were performed on ice or at 4°C. Protein concentrations were determined by Bradford assay.

Gel retardation assays were performed as previously described (18) with some modifications. Nuclear extracts (1 to 5 μg) were incubated with 1 μg of poly(dI-dC) and 3 μl of 5× DNA binding buffer (10% [wt/vol] polivinylethanol, 12.5% [vol/vol] glycerol, 50 mM Tris [pH 8], 2.5 mM EDTA, 2.5 mM DTT) in a final volume of 13 μl on ice for 10 min. Next, 25,000 cpm (2.5×10^7 to 5×10^7 cpm/μg) of ^{32}P-labeled double-stranded oligonucleotides (2 μl) was added, and this mixture was incubated at room temperature for 40 min. In competition experiments, 30-fold molar excess of unlabeled homologous oligonucleotides was added to the binding reaction mixture prior to the addition of the probe. The DNA-protein complexes were resolved by electrophoresis on a 4% nondenaturing polyacrylamide gel. The sequences of the oligonucleotides (5′ to 3′) used as probes in EMSAs were gatcGGAGGAAAAACTGTTTCATACAGAAGGCGT (distal NFAT site of human IL-2 promoter), gatcGGGATTTCACCT (NF-κB binding site of human IL-2 promoter), and GCCCCCTCTGACTCATGCTG ACA (nucleotides −68 to −46, including the AP-1 site of the *CD11c* promoter).

The pairs of complementary synthetic oligonucleotides were annealed and labeled with the Klenow fragment of the DNA polymerase I (for NF-κB and NFAT oligonucleotides) or with the avian myeloblastosis virus reverse transcriptase in the case of AP-1 oligonucleotide.

Supershift assays were performed by incubating the antiserum (0.5 μl) and nuclear extract for 15 min at 4°C, before addition of the probe to the binding reaction mixture. The antiserum 67.1, specific for NFAT1/p, has been previously described (21).

Western blot analysis. After the different treatments, cells (2×10^6) were washed with ice-cold PBS and resuspended in hypotonic buffer (10 mM Tris-Cl [pH 7.5], containing 10 mM NaCl, 3 mM $MgCl_2$, 1 mM PMSF, 0.5 mM DTT, 0.1 mM EGTA, 2 μM leupeptin, 1 μg of aprotinin per ml, and 0.05% Nonidet P-40). Cells were then centrifuged at 650 × *g* to pellet the nuclei, and the supernatant was removed. The nuclear pellet was washed in the hypotonic buffer without detergent and resuspended in Laemmli buffer. For the preparation of whole-cell extracts, total proteins from the different cells (10^6) were extracted as previously described (2). Nuclear and total extracts were boiled and separated by sodium dodecyl sulfate (SDS)-polyacrylamide (6%) gel electrophoresis under reducing conditions. Gels were transferred to nitrocellulose membranes that were incubated in blocking solution (5% [wt/vol] skim milk in Tris-buffered saline [TBS] buffer) for 90 min at room temperature, washed twice in TBS-T (TBS, 0.05% Tween 20), and incubated with antiserum 67.1 (0.03% [vol/vol]) in TBS-T for 2 h. Membranes were then washed four times for 5 min each in TBS-T, and peroxidase-labeled goat anti-rabbit IgG (Pierce) was incubated for 90 min at room temperature. After three washes with TBS-T and one wash with H_2O, membrane-bound antibody was visualized with the Amersham ECL (enhanced chemiluminescence) detection reagent.

Solid-phase JNK assays. After the indicated treatment, PBLs (5×10^6 per sample) were lysed for 15 min on ice with lysing buffer (20 mM HEPES [pH 7.6],

10 mM EGTA, 40 mM β-glycerophosphate, 1% Nonidet P-40, 2.5 mM $MgCl_2$, 2 mM sodium orthovanadate, 1 mM DTT, 1 mM PMSF, 20 μg of aprotinin per ml, 20 μg of leupeptin per ml) and centrifuged at 12,000 × *g* for 10 min at 4°C. The supernatants were collected and incubated with 0.3 μg of a rabbit polyclonal anti-JNK-1 antibody (Santa Cruz Biotech, Inc.). One hour after this incubation, protein A beads were added, and the mixture was incubated at 4°C for an additional 2 h. The immune complexes were washed three times with PBS that contained 1% Nonidet P-40 and 2 mM vanadate and once with kinase buffer (20 mM HEPES [pH 7.6], 2 mM DTT, 10 mM β-glycerophosphate, 20 mM $MgCl_2$, 0.1 mM Na_3VO_4). After the washing, the supernatant was removed and the kinase reaction was initiated by addition of 40 μl of kinase buffer containing 20 μM cold ATP, 0.1 μCi of [γ-^{32}P]ATP, and 1 μg of GST-c-Jun(1–79) (GST is glutathione *S*-transferase) or GST-NFATp(1–415) per reaction at 30°C for 20 min. The reaction was stopped by addition of 10 μl of 5× Laemmli buffer, and this mixture was boiled for 5 min and then analyzed by SDS gel electrophoresis on a 12% acrylamide gel. GST-c-Jun(1–79) and GST-NFATp(1–415) proteins were isolated from 250 ml of bacterial cultures expressing pGEX-c-Jun(1–79) plasmid (19) or pGEX2T-NFATp(1–415) (33) plasmid, respectively, with GSH-Sepharose 4B beads (Pharmacia Biotech., Inc.).

Northern blot analysis. After different treatments, Jurkat cells were harvested, and total RNA was isolated with the Ultraspec system (Biotecx Laboratories, Inc.). Poly(A)$^+$ RNAs were purified by using the PolyATtract mRNA isolation system III (Promega). For Northern blot analysis, poly(A)$^+$ RNA from each sample (0.5 μg) was denatured, electrophoresed on a 1% formaldehyde agarose gel, and blotted onto a nitrocellulose membrane. After UV cross-linking, the filters were hybridized overnight at 42°C with the corresponding specific probes: a 2.2-kb *Eco*RI fragment of the NFATc cDNA (40), a 0.8-kb *Pst*I fragment of IL-2 cDNA, a 0.8-kb *Hin*dIII-*Pst*I fragment of c-Jun cDNA, and a 0.6-kb *Hin*dIII-*Bam*HI fragment of the β-actin cDNA.

Immunocytochemical localization of NFATp and NFATc. Jurkat cells (10^7) were electroporated with 10 μg of pEF-BOS NFAT1 plasmid, which expresses influenza virus hemagglutinin (HA)-tagged NFATp (33), or 20 μg of pSH102CΔ418 (provided by G. Crabtree), which expresses HA-tagged NFATc (1 to 418) (43). Forty-eight hours after transfection, cells were plated on polylysine-coated coverslips and either were left untreated or were incubated with PDTC (50 μM) for 2 h before stimulation with PMA plus Ca^{2+} ionophore as indicated. The subcellular localization of NFATp was analyzed by immunofluorescence with anti-HA antibody (12CA5) as previously described (33). The percentages of cells displaying nuclear or cytoplasmic staining were scored visually after counting of at least 150 HA-expressing cells in the experiments shown. Two or four different fields are presented at each time point analyzed.

RESULTS

Inhibition of T-cell activation by DTCs. Signals transduced by the activation of T-cell receptor (TCR) induce the synthesis of IL-2 and the expression of IL-2 receptors (IL-2Rs) and other activation antigens (14, 70). The activation process can be mimicked by simultaneous treatment of T cells with stimuli that activate protein kinase C and elevate intracellular Ca^{2+}, such as phorbol esters and calcium ionophores (15, 67). To determine the effects of the different antioxidants on the T-cell activation process, flow cytometry analysis of Jurkat cells activated with the phorbol ester PMA and the calcium ionophore A23187, either preincubated or not with different DTCs, PDTC, or DDTC and its disulfide-linked form disulfiram, were carried out. As shown in Fig. 1, doses of DTCs in the range of 50 to 100 μM inhibited the upregulated expression of the T-cell activation antigen CD69 and the IL-2Rα chain (CD25) induced by PMA plus ionophore. The pretreatment with DTCs strongly inhibited the cell surface expression of CD25, whereas that of CD69 was not totally blocked. Conversely, DTCs induced low levels of expression of CD69 that persisted but were not further increased by subsequent stimulation with PMA plus ionophore. Similar results were obtained by using unfractionated PBLs stimulated with PMA plus ionophore, and inhibition of CD69 membrane expression was also observed in the T-cell population activated through the CD3-TCR complex (Fig. 1B).

The synthesis of IL-2 requires the efficient integration of signals transmitted through different pathways during T-cell activation. We next explored whether the inhibitory effects observed with the DTCs were also reflected at the IL-2 expression level. The IL-2 secretion by Jurkat cells in response to

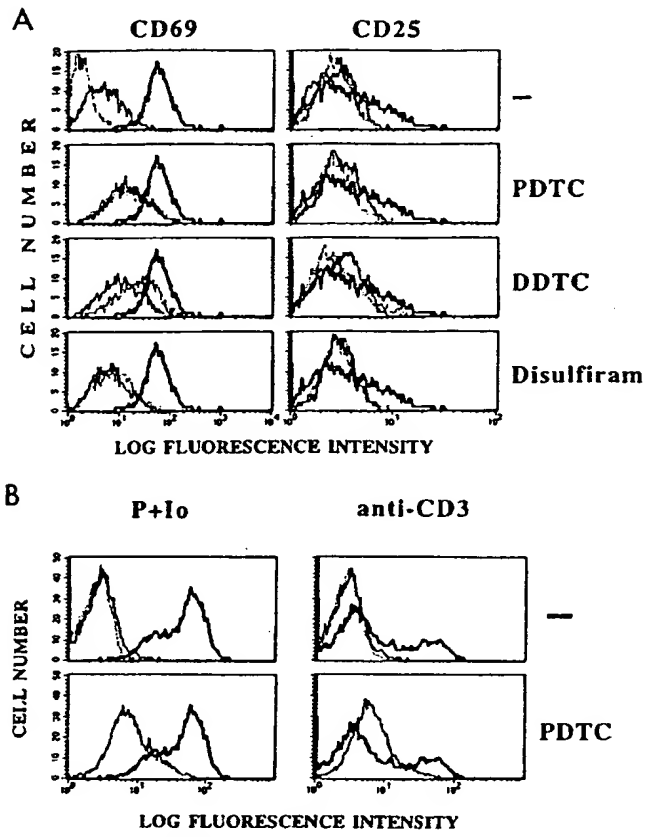

FIG. 1. Effect of DTCs on membrane expression of T-cell activation markers. (A) Flow cytometry profiles of CD69 and CD25 expression in Jurkat cells either untreated or treated with different DTCs (thin lines) are compared with those of cells stimulated with PMA plus Ca^{2+} ionophore (thick lines) or cells pretreated with the DTCs for 2 h and further stimulated with PMA plus ionophore (dotted lines). In the control panel (–) dotted lines indicated the staining with P3X63 myeloma supernatant, used as a negative control, and thin lines indicate the baseline expression of CD69 or CD25. DTCs were added for 16 h at doses of 100 μM (PDTC and DDTC) and 50 μM (disulfiram). PMA (20 ng/ml) and Ca^{2+} ionophore A23187 (1 μM) were also added for 16 h. (B) Profiles of CD69 expression in PBLs untreated or pretreated for 2 h with 50 μM PDTC and further stimulated with 20 ng of PMA per ml plus 1 μM ionophore or by incubation in UCHT.1 (anti-CD3)-coated cells (thin lines) are compared with those of cells stimulated with PMA plus ionophore or immobilized anti-CD3 antibody (thick lines). Cells were treated with the indicated reagents for 16 h. The dotted lines indicated the staining with anti-ELAM antibody used as a negative control.

PMA plus ionophore was inhibited, in a dose-dependent manner, by the different antioxidants tested (Fig. 2). DTCs also blocked IL-2 secretion at concentrations ranging from 20 to 50 μM. Since DDTC and disulfiram, but not PDTC, have been reported to produce acidification in long-term-treated cell cultures (53) and PDTC displayed a very potent inhibitory effect on membrane expression of T-cell activation markers and IL-2 secretion, we used this derivative of DTC to further characterize the mechanism accounting for the interference with the T-cell activation process observed.

Blockade of IL-2 transcription by DTCs. To characterize the molecular mechanisms involved in the inhibition of the IL-2 secretion by DTCs, we performed Northern blot analysis of Jurkat cells treated or not with PDTC and then stimulated with PMA plus ionophore. IL-2 mRNA steady-state levels, detected as early as 4 h and strongly increased after 8 h of stimulation, were totally abrogated in cells exposed to PDTC (Fig. 3A). The

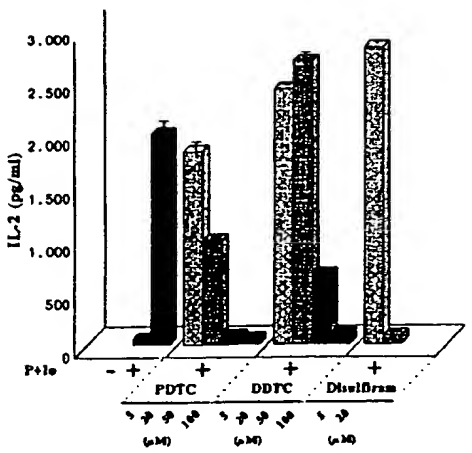

FIG. 2. Inhibition of IL-2 production by DTCs. Jurkat cells were either pretreated or not with the indicated micromolar concentrations of DTCs for 2 h and then were stimulated with 20 ng of PMA per ml plus 1 μM Ca^{2+} ionophore A23187 (P+Io). IL-2 was measured in the supernatants by enzyme-linked immunosorbent assay 20 h after stimulation. The levels of IL-2 detected in supernatant of cells single treated with the different DTCs were the same as those in the control cells (≤60 pg/ml). The results are represented as the mean ± standard deviation of two independent measures. Cell viability, tested by trypan blue exclusion just before collection of the supernatants, was higher than 90% for the different treatments.

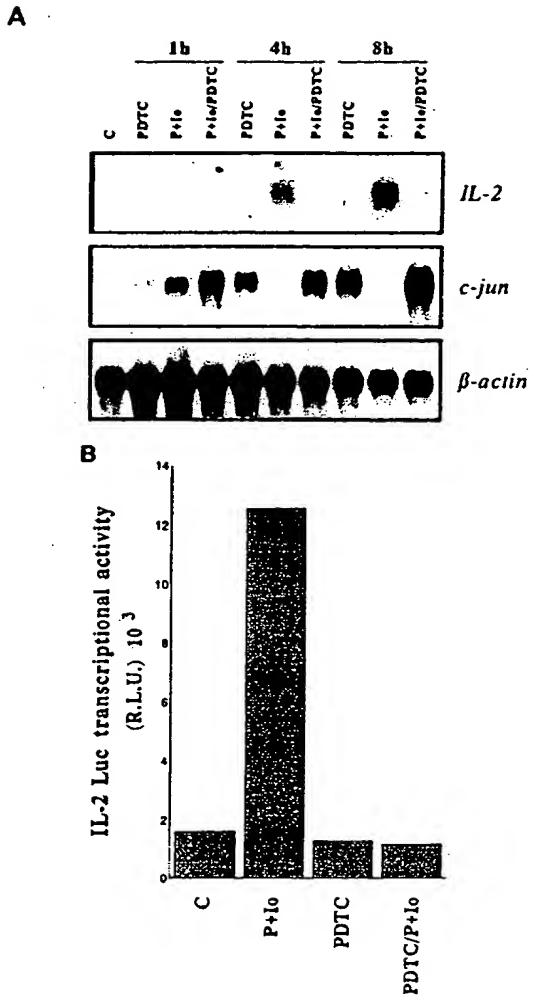

FIG. 3. Effects of PDTC on IL-2 transcription. (A) Northern blot analysis with poly(A)$^+$ RNAs from Jurkat cells treated with PDTC or PMA plus A23187 (P+Io) or pretreated with PDTC for 2 h and then stimulated with PMA plus A23187 (P+Io/PDTC) for the times indicated. After agarose electrophoresis and blotting, the membrane was sequentially hybridized with IL-2, c-Jun, and β-actin probes as indicated. C, control. (B) The transcriptional activity of IL-2 promoter (bp −326 to +45) was tested by transfection of Jurkat cells with Lipofectin for 8 h. Thirty-six hours posttransfection, cells were treated with PDTC or PMA plus ionophore (P+Io) or were preincubated with PDTC for 2 h and further stimulated with PMA plus ionophore (PDTC/P+Io) for 8 h. The results are expressed as relative light units (R.L.U.) measured for 30 s. Results are representative of five independent experiments. PDTC (50 μM), PMA (20 ng/ml), and Ca^{2+} ionophore (1 μM) were used at the same doses in single or combined treatments in panels A and B.

treatment with PDTC did not result in nonspecific or toxic inhibition of gene expression; rehybridization of the blotted membrane with a c-jun probe revealed that PDTC triggered a simultaneous and sustained induction of the c-jun transcripts, as previously described (18). Furthermore, c-jun mRNA levels were synergistically increased by treatment with PDTC plus the combination of PMA and ionophore under conditions in which IL-2 transcription was completely abolished (Fig. 3A). Since the proximal region of the IL-2 promoter (containing 275 bp of the upstream regulatory region of the IL-2 gene) includes binding sites for a number of transcription factors that mediate the induction of IL-2 gene expression (15), we next studied whether the inhibitory effects of DTCs on IL-2 transcriptional expression were mediated through inhibition of the IL-2 promoter. The transcriptional activity of Jurkat cells transfected with a reporter plasmid containing the IL-2 promoter region in response to PMA plus ionophore was abrogated by treatment with PDTC (Fig. 3B), DDTC, or disulfiram (data not shown).

Effects of PDTC on different transcription factors that regulate IL-2 promoter activity. We further dissected the mechanisms responsible for the PDTC-mediated inhibition of IL-2 transcription by analyzing the effects of the DTC on the activity and binding of the transcription factors AP-1, NF-κB, and NFAT, which have been shown to be critical for the activity of the IL-2 promoter (15, 26, 46). As described for other stimuli (52, 53), the potent activation by PMA plus ionophore of the NF-κB reporter construct was blocked by pretreatment with PDTC (Fig. 4A, upper). In contrast, the activity of an AP-1-dependent reporter plasmid, which is stimulated by PDTC in Jurkat cells (18), was synergistically increased by the combination of PMA plus ionophore and PDTC (data not shown). Although AP-1 cooperates with NFAT to activate transcription through the NFAT distal site of the IL-2 promoter, interestingly, the transcriptional activation of a reporter construct directed by this NFAT motif was abrogated by PDTC (Fig. 4A, lower). The data obtained in these transfection experiments correlated with the effect of PDTC on the DNA binding of such transcription factors analyzed by EMSAs. Thus, the specific binding to NF-κB and NFAT probes from the human IL-2 promoter, strongly induced by PMA plus ionophore, was inhibited by DTC, whereas AP-1 DNA-binding activity was stimulated by PDTC with the same nuclear extracts (Fig. 4B). These effects were not due to a direct interference of PDTC with DNA-transcription factor interaction, since exogenous addition of PDTC to the binding reaction mixtures in EMSAs, even at concentrations of 100 μM, did not affect the formation of the specific complexes (data not shown). The effect of PDTC on NF-κB DNA-binding activity was also observed in PBLs activated by PMA plus ionophore (data not shown). In addition, PDTC completely blocked the specific binding to the

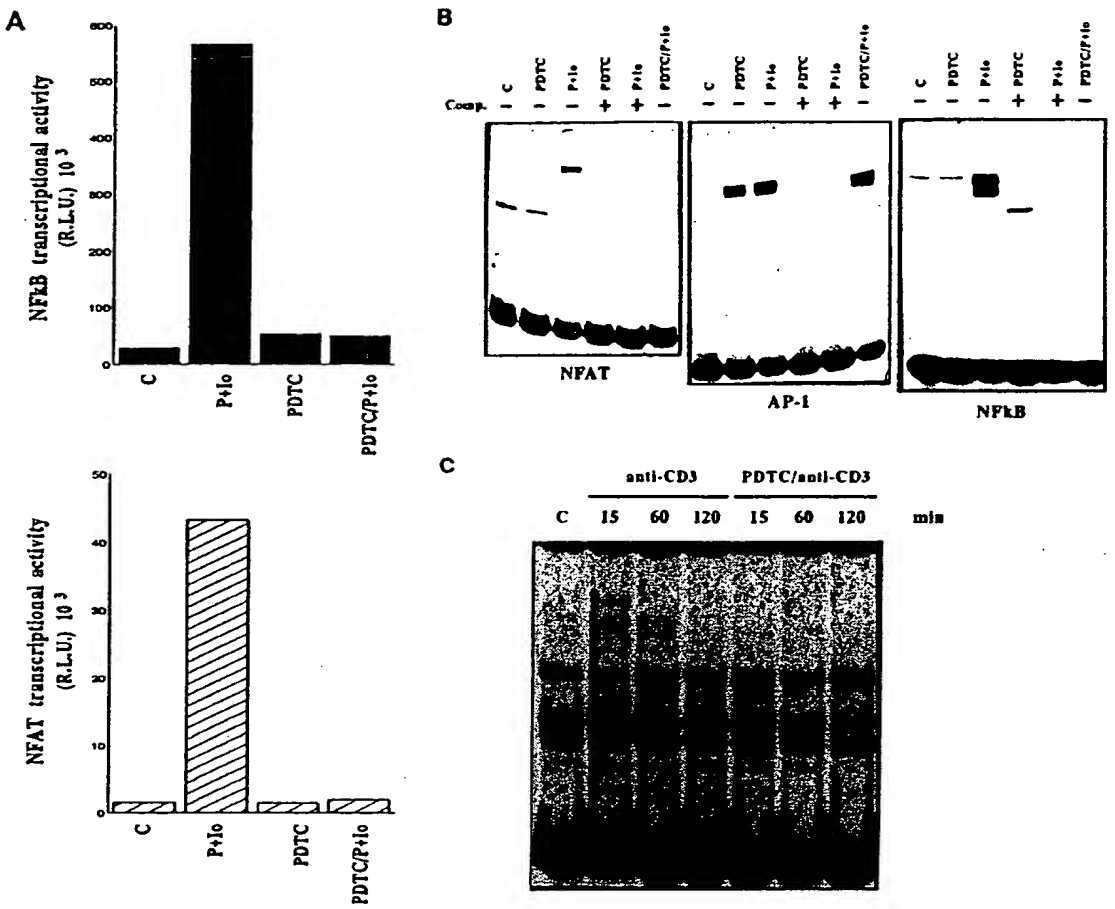

FIG. 4. PDTC-mediated changes in the activity and DNA binding of transcription factors that regulate IL-2 promoter activity. (A) Transcriptional activities of NF-κB- and NFAT-dependent luciferase reporter plasmids were analyzed by transfection of Jurkat cells. Cells were transfected and treated with PDTC or PMA plus ionophore or were pretreated with PDTC and then stimulated with PMA plus ionophore with the same doses and conditions described in the legend to Fig. 3. (B) DNA-binding activities of NFAT, AP-1, and NF-κB factors were analyzed with the same pools of nuclear extracts from Jurkat cells either untreated or treated with 100 μM PDTC or 20 ng of PMA per ml plus 1 μM ionophore (P+Io) for 4 h. For the combined treatment (PDTC/P+Io), PDTC was incubated for 2 h, followed by a 4-h stimulation with P+Io at the same doses as those used in separate treatments. Specific binding was tested by addition to the binding reaction mixtures of 30-fold molar excess of unlabeled homologous oligonucleotide (Comp. +). (C) NFAT DNA-binding activity was analyzed for nuclear extracts from purified T lymphocytes stimulated with cross-linked T3b anti-CD3 antibody for 15, 60, and 120 min. Cells were pretreated with PDTC for 2 h and further stimulated with anti-CD3 antibody (PDTC/anti-CD3) for the indicated times.

NFAT probe in nuclear extracts from both PBLs activated by PMA plus ionophore and T cells stimulated by cross-linking with immobilized T3b anti-CD3 MAb (Fig. 4C and data not shown).

Mechanisms of inhibition of NFAT by PDTC. The possible effects of DTC on the activity of the NFAT cytosolic component that could account for the observed inhibition were analyzed. Other drugs which block the binding and transactivating abilities of NFAT, such as CsA and FK 506, target the phosphatase activity of calcineurin, thus preventing the subsequent dephosphorylation and translocation of cytosolic NFAT (28, 55). Therefore, we explored whether PDTC affected the dephosphorylation of NFAT1/p and its translocation to the nucleus upon activation of Jurkat cells. Western blot analysis of fractionated cellular extracts indicated that treatment with PMA plus ionophore or ionophore alone induced the dephosphorylation and translocation to the nucleus of NFAT. As previously reported (29, 49, 60, 65), the dephosphorylation or translocation of NFAT was detected as early as 5 min, maintained for at least 90 min, and blocked by CsA (Fig. 5A). Strikingly, exposure of cells to PDTC prior to the addition of ionophore or PMA plus ionophore resulted in transient dephosphorylation and translocation of NFATp, and the bulk of the transcription factor was found in the cytosolic extracts, after 90 min of single or combined treatment with PDTC (Fig. 5A and data not shown). It is noteworthy that the mobility of NFATp exported from the nucleus of PDTC-treated cells was lower than that displayed by the phosphorylated form in untreated Jurkat cells (Fig. 5A). To further analyze the effects of PDTC on the nuclear shuttling of NFATp, we performed immunocytochemical experiments in Jurkat cells transiently transfected with HA-tagged full-length NFATp (33). As shown in Fig. 5B, HA-tagged recombinant NFATp was found in the cytoplasm of unstimulated cells and translocated to the nucleus upon stimulation with PMA plus ionophore. In agreement with the Western blot analysis, pretreatment with PDTC promoted a rapid nuclear export of NFATp from the nucleus of cells activated with PMA plus ionophore. In these cells, the tagged recombinant NFATp protein was already detected in the cytoplasm of ≈50% of the transfected cells after 30 min of activation, and the nuclear export was complete by 90 min (Fig. 5B, lower panel). Therefore, PDTC, by mechanisms at present

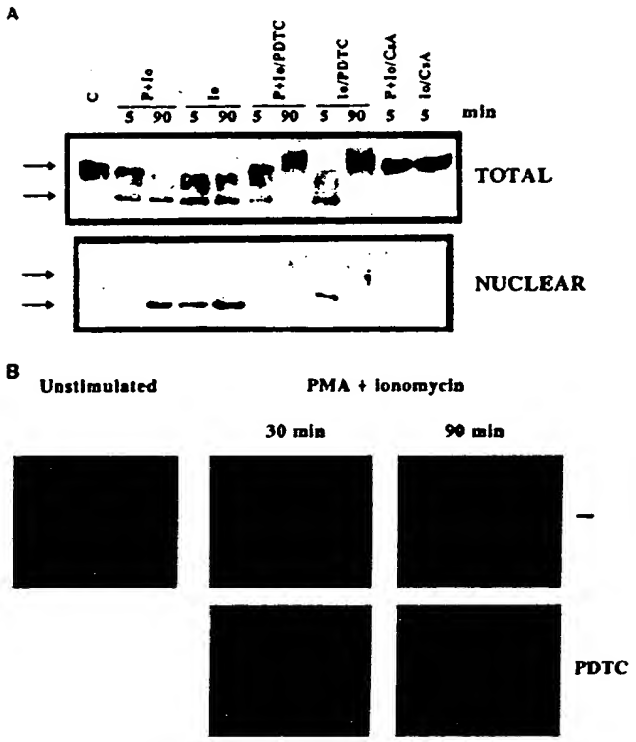

FIG. 5. Rapid shuttling of NFATp induced by PDTC. (A) Western blot analysis of fractionated cell extracts from Jurkat cells (10^6 per line in nuclear lysates and 5×10^5 for total lysates) treated for 5 or 90 min with 20 ng of PMA per ml plus 1 μM ionophore or 1 μM ionophore alone either pretreated or not with PDTC (100 μM) or CsA (100 ng/ml) for 2 and 1 h, respectively. The antiserum 67.1 was used for NFAT1/p detection. The electrophoretic mobilities of the upper and lower bands corresponding to the phosphorylated and dephosphorylated forms of NFAT1, respectively, are indicated by arrows. C, control. (B) Immunocytochemical staining of Jurkat cells transfected with HA-tagged NFATp either untreated (−) or incubated with PDTC (50 μM) for 2 h. After incubation, cells were stimulated with PMA (20 ng/ml) plus ionomycin (3 μM) (PMA+ionomycin) for 30 or 90 min, fixed, and analyzed by immunofluorescence with anti-HA antibody. The upper panel shows the subcellular localization of HA-tagged NFATp in resting and stimulated cells, and the lower panel corresponds to stimulation with PMA plus ionomycin in PDTC-pretreated cells.

unknown, accelerates the shuttling of the transcription factor in activated Jurkat cells.

In view of the requirement of sustained activation to promote NFAT-dependent transcription (65), we next investigated whether PDTC affected the transactivating ability of NFAT in activated cells. For this purpose, we performed cotransfection experiments with a chimeric expression plasmid encoding the NH_2-terminal transactivation domain of NFATp fused to the GAL4 DBD (32), together with a GAL4-Luc reporter plasmid. As shown in Fig. 6, PDTC prevented the transactivation function of NFATp induced by PMA plus ionophore. As controls, in parallel transfections, we included GAL4 expression vectors encoding the transactivation domain of c-Jun (GAL4–c-Jun wild type). Strikingly, PDTC inhibited the transactivation by the GAL4-NFAT hybrid induced by PMA plus ionophore, under the same conditions that strongly increased the transactivation by the GAL4-c-Jun construct. As occurred with the activity of an AP-1 reporter construct, PDTC and PMA-ionophore strongly synergized activating the GAL4–c-Jun construct. As an additional control, we also included in the experiments a plasmid identical to the GAL4–c-Jun wild type but double mutated in serines located at positions 63 and 73 (GAL4–c-Jun $S_1 + S_2$) (45). Transactivation by this mutated plasmid was not affected at all by treatments with the stimuli that strongly increased the activity of the wild type (Fig. 6).

In order to investigate whether the effect of PDTC on the rapid nuclear export of NFATp in activated Jurkat cells was also operative in other cell types, we carried out Western blotting experiments with the γδ T-cell line PEER as well as with PBLs. The phosphorylated status of NFATp was monitored after different times of activation with ionophore in fractionated cellular extracts of control and PDTC-treated cells. As shown in Fig. 7, pretreatment of PBLs and PEER cells with PDTC resulted in a rapid export of NFATp with a kinetics similar to that displayed by Jurkat cells. Similarly, treatment with the Ca^{2+} ionophore induced the sustained presence of the dephosphorylated form in the nucleus for at least 90 min in these cells.

Since DTCs are able to activate JNK (18) and NFAT kinases have been proposed to be involved and implicated in the activation of the nuclear export of NFAT-4 and NFATc, respectively (5, 61), we next analyzed whether NFATp was a target for JNK. Solid-phase kinase analysis of JNK immunoprecipitated from PBLs treated with either PMA plus ionophore or PDTC indicated that both stimuli activated JNK activity, inducing the phosphorylation of both NFATp and c-Jun (Fig. 8). This striking result shows that NFATp is a substrate of JNK, which is activated after PBL treatment with PDTC or PMA plus ionophore.

Recent studies have shown that the composition of NFAT changes during the T-cell activation process. Thus, NFATp is rapidly activated and translocated to the nucleus within minutes after T-cell activation. Several hours after activation, the bulk of NFATp is found in the cytoplasm, and other NFAT family members have been suggested to mediate NFAT-dependent sustained transcription (29). In addition, in NFAT1/p-deficient mice, IL-2 gene expression upon signaling through the TCR does not appear to be significantly affected (22, 72), which suggests that other NFAT family members might play a more relevant role in IL-2 gene activation or compensate for the absence of NFAT1/p. Since we had observed complete inhibition of NFAT-dependent transcription by PDTC, we decided to analyze by EMSA its effects on the kinetics of binding of NFAT in Jurkat cells activated with PMA plus ionophore. These experiments showed that nuclear factor binding to the NFAT probe, observed after 1 h of activation and further increased after 4 and 8 h, was inhibited by PDTC at all different times analyzed (Fig. 9, left). Serological analysis with the 67.1 specific anti-NFATp antibody revealed the predominant presence of NFATp in nuclear extracts of cells activated for 1 h, while 8 h after activation, excess of the specific antiserum only blocked and supershifted part of the NFAT complex (Fig. 9, right). Since NFATc has been shown to be transcriptionally induced during T-cell activation (43) and is detected in the nucleus later after T-cell stimulation (29), we performed Northern blot analysis to determine whether the expression of NFATc during activation was affected by PDTC. These experiments revealed that the NFATc mRNA steady-state levels, undetected in untreated Jurkat cells, were upregulated after 1 h of treatment with PMA plus ionophore, peaked at 4 h, and were maintained at high levels for at least 8 h of activation. Exposure to PDTC prior to the activation of the cells drastically inhibited NFATc gene expression at the times analyzed (Fig. 10A and data not shown).

To determine whether nuclear export of NFATc was affected by PDTC, we performed immunofluorescence analysis of Jurkat cells transfected with HA-tagged NFATc(1–418).

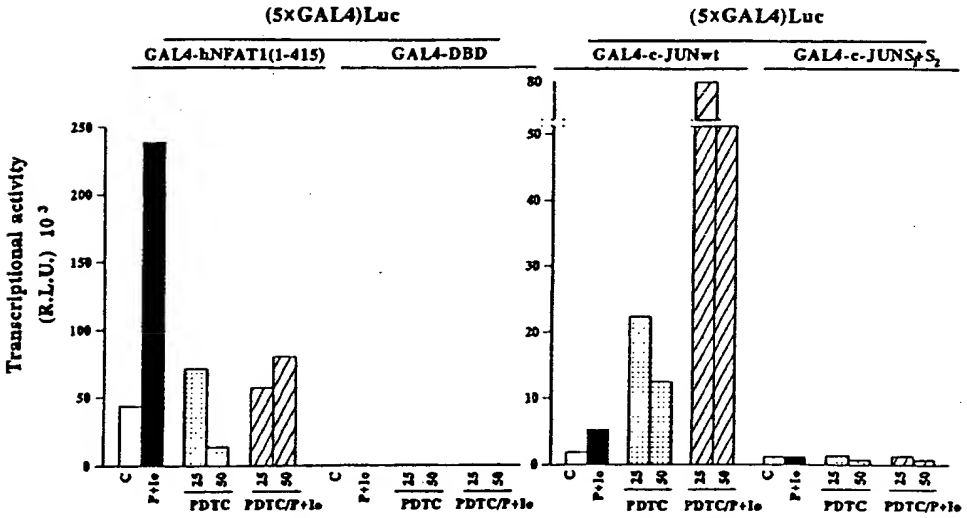

FIG. 6. Opposite effects of PDTC on the transactivation of NFATp and c-Jun. Jurkat T cells were cotransfected by lipofection with 4 μg of a GAL4-Luc reporter plasmid per ml together with 2 μg of the expression vectors coding for the fusion proteins GAL4-hNFAT1(1–415); GAL4–c-Jun (wt, wild type); the mutated version, GAL4–c-Jun S_1 + S_2; or the parental empty vector RSV-GAL4-DBD per ml for 8 h. After 36 h, cells were pretreated or not with 25 or 50 μM PDTC and further stimulated with 20 ng of PMA per ml plus 1 μM A23187 for 3 h. The results are expressed as relative light units (R.L.U.) measured for 30 s and are representative of three independent experiments.

These experiments revealed that the translocation of recombinant NFATc to the nucleus was detected as early as 15 min in 30 to 50% of the activated cells either treated or not with PDTC. However, after 90 min, the nuclear export was total in the cells treated with the DTC, whereas the staining pattern remained nuclear in 90% of the cells activated in the absence of PDTC (Fig. 10B).

Taken together, our results indicate that the combined effects of PDTC inhibiting the transactivating functions of NFATp and the transcriptional induction of NFATc might well account for the inhibition of binding and NFAT reporter-dependent transcription of activated T cells. In addition, given the critical role of NFAT in the IL-2 gene regulation (15, 46), this combined effect could itself account for the inhibition of IL-2 transcription and expression.

DISCUSSION

The transmission of signals derived from the T-cell stimulation involves at least three different pathways mediated by Ras, protein kinase C, and Ca^{2+}-calcineurin (15, 24). These pathways couple the signals from the plasma membrane with the activation of transcription factors which regulate the expression of a large number of genes during the T-cell activation program. Among these genes, those that code for cytokines play a crucial role in the regulation of the immune response. Cytokines control processes such as proliferation and differentiation, as well as multiple effector functions of immune cells. The fact that the immunosuppressive drugs CsA and FK 506 abrogate the cytokine transcription emphasizes not only the relevance of cytokines in regulating the immune responses, but also the central role that the Ca^{2+}-calcineurin pathway plays in T lymphocytes. In this study, we showed that different DTCs interfere with the T-cell activation process and analyzed the effect of the pyrrolidine derivative of DTC (PDTC) on the transcription factors that regulate IL-2 gene expression. This analysis revealed that PDTC strongly inhibited NFAT activation by mechanisms different from those used by the immunosuppressive drugs CsA and FK 506.

We have shown that DTCs block IL-2 production and inhibit the surface upregulation of T-cell activation antigens such as CD69 and CD25 in both Jurkat and peripheral blood T cells.

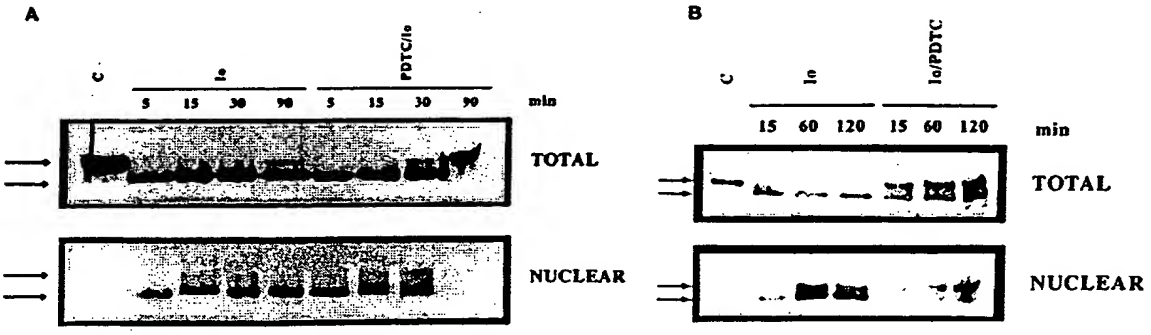

FIG. 7. Shuttling of NFATp in PBLs and in the PEER T-cell line. Western blot analysis of fractionated cell extracts from PEER cells (A) or PBLs (B) (5×10^5 cells per sample in total and 10^6 cells for nuclear lysates) treated for the indicated times with 1 μM ionophore (Io) either pretreated or not with 100 μM PDTC for 2 h. For NFAT1/p detection, the antiserum 67.1 was used. The arrows indicate the phosphorylated and dephosphorylated forms of NFATp. C, control.

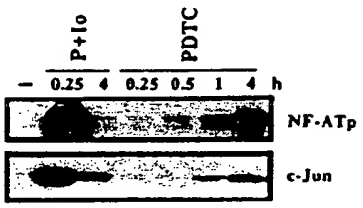

FIG. 8. JNK phosphorylates NFATp in vitro. PBLs treated with 20 ng of PMA per ml plus 1 μM ionophore (P+Io) or 100 μM PDTC were lysed and immunoprecipitated with antibody specific to JNK1 at the indicated times. The immune complexes were incubated with GST–c-Jun(1–79) or GST-NFATp(1–415) in kinase buffer in the presence of [γ-^{32}P]ATP for 20 min. The reactions were stopped and separated on SDS–12% polyacrylamide gels.

Although these effects clearly reflect inhibition of the T-cell activation process, DTCs by themselves also trigger signals leading to a moderate surface expression of CD69. In fact, the expression of CD69 in Jurkat cells stimulated with PMA plus ionophore or in T cells activated with anti-CD3 MAb was not completely inhibited by DTCs and remained at the same level as those induced by the single treatment with the DTCs. This activating effect resembles that exerted by PDTC on ICAM-1 activation in endothelial cells (40) and appears to be mediated by transcriptional induction of the CD69 gene promoter through AP-1 (9a). Given the fact that PDTC is able to induce a sustained and strong activation of JNK in Jurkat T cells (18) as well as in human PBLs (Fig. 8), such stimulation may also be related to the observed CD69 upregulation.

Despite its ability to activate AP-1 in several cell types, including T cells (18, 57), DTCs have been proposed as potential inhibitors of cytokine production in T cells by virtue of their capacity to block NF-κB activation (52). The metal-chelating and oxygen radical-scavenging antioxidative properties of these compounds have been suggested to mediate the inhibition of this transcription factor (52). Strikingly, as occurs with DTCs, other antioxidants such as NAC and thioredoxin not only are inhibitors of NF-κB, but also activate AP-1 (38, 51, 52, 63). Although we have observed that the antioxidants NAC and BHA also interfere with the T-cell activation process, their inhibitory effects on the expression of activation antigens and IL-2 production are weaker than those displayed by DTCs (data not shown). In addition, we have found that NAC fails to interfere with the nuclear shuttling of NFATp upon activation of Jurkat cells (data not shown). Thus, the effects of NAC on T-cell activation might be more related to its capacity to inhibit NF-κB. Although these experiments suggest that the effects of DTCs on NFAT are not related to their reactive oxygen intermediate scavenger properties, we cannot exclude the possibility that the stronger antioxidative potency of DTCs could account for such effects.

The relative contributions of different members of the NFAT family of transcription factors to the transcriptional activation of the IL-2 gene are not completely understood. It seems that NFATp plays a major role in early T-cell activation and then is slowly deactivated (29), whereas the bulk of NFATc is induced and activates transcription later on. However, in NFAT1/p-deficient mice, IL-2 transcription upon signaling through the TCR is not significantly affected (22, 72), which suggests that other NFAT family members may play a more important role in the transcriptional regulation of the IL-2 gene. Thus, the inhibitory effects of PDTC on NFATc may be more relevant to the inhibition of IL-2 than that exerted on the transactivating abilities of NFATp. The prominent role of NFATc in this process would also be consistent with the severe inhibition that the expression of a transdominant negative of NFATc exerts on the transcriptional activation of the IL-2 promoter construct (reference 43 and data not shown). Although we have not addressed the mechanisms by which PDTC blocks NFATc gene expression, it is tempting to speculate that the transcription factors inhibited by PDTC would also be involved in the transcriptional regulation of NFATc. In such case, although we do not rule out that DTCs may affect other transcription factors, NF-κB or NFAT family members would be good candidates for regulation of NFATc gene transcription. Initially, the presence of normal levels of NFATc in NFATp-deficient mice would argue against an essential role of NFATp in the regulation of NFATc expression. However, a putative role of NFATp in regulating NFATc-inducible expression cannot be excluded, and compensatory mechanisms may also operate through other family members, such as NFATx, in the deficient mice. The functional characterization of the *cis*-acting and *trans*-acting elements involved in the transcriptional regulation of the NFATc gene promoter will clarify which factors are actually involved in this regulation.

In contrast to the sustained translocation to the nucleus induced by ionophore or PMA plus ionophore (29), PDTC triggered a rapid nuclear shuttling of NFATp in the different T-cell types analyzed. Recently, calcium signaling has been shown to induce an association between the NFAT4 family member and calcineurin, which are both imported to the nucleus as a complex. Once in the nucleus, a putative kinase has been proposed to promote the nuclear export of the transcription factor (61). Furthermore, glycogen synthase kinase-3 has been shown to phosphorylate critical serines of NFATc that are required for nuclear export and to enhance the nuclear exit of the transcription factor. However, glycogen synthase kinase-3 activity requires the previous phosphorylation of NFATc by an unknown kinase(s) that could directly phosphorylate NFATc (5). In this context, it is tempting to speculate about the existence of an NFAT kinase activated by PDTC that would be responsible for the accelerated induction of the nuclear export of NFAT. Supporting this hypothesis is the fact that NFATp exported from the nucleus of PDTC-treated cells

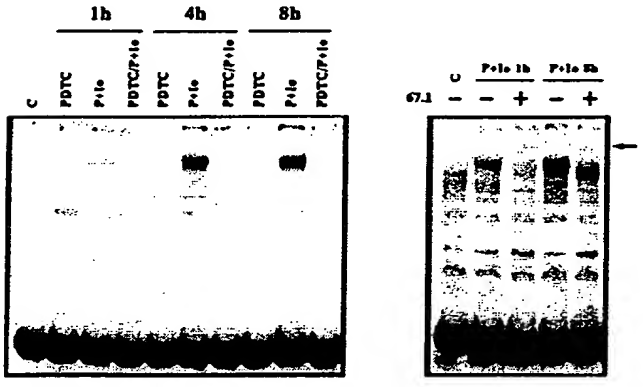

FIG. 9. Kinetics analysis and contribution of NFATp to DNA-binding activity of NFAT. DNA-binding activity of NFAT was analyzed in nuclear extracts from Jurkat cells treated for 1, 4, and 8 h with PDTC or PMA plus ionophore (P+Io) or preincubated for 2 h with PDTC and then treated with PMA plus ionophore for different times as indicated (left panel). C, control. Nuclear extracts from Jurkat cells stimulated with the PMA plus ionophore for 1 and 8 h were analyzed by EMSA in the presence or absence of 0.5 μl of the anti-NFATp antibody 67.1. The autoradiograph was overexposed to visualize the supershifted complex, indicated by an arrow (right panel). Doses of 100 μM PDTC, 20 ng of PMA per ml, and 1 μM A23187 were used in single or combined treatments.

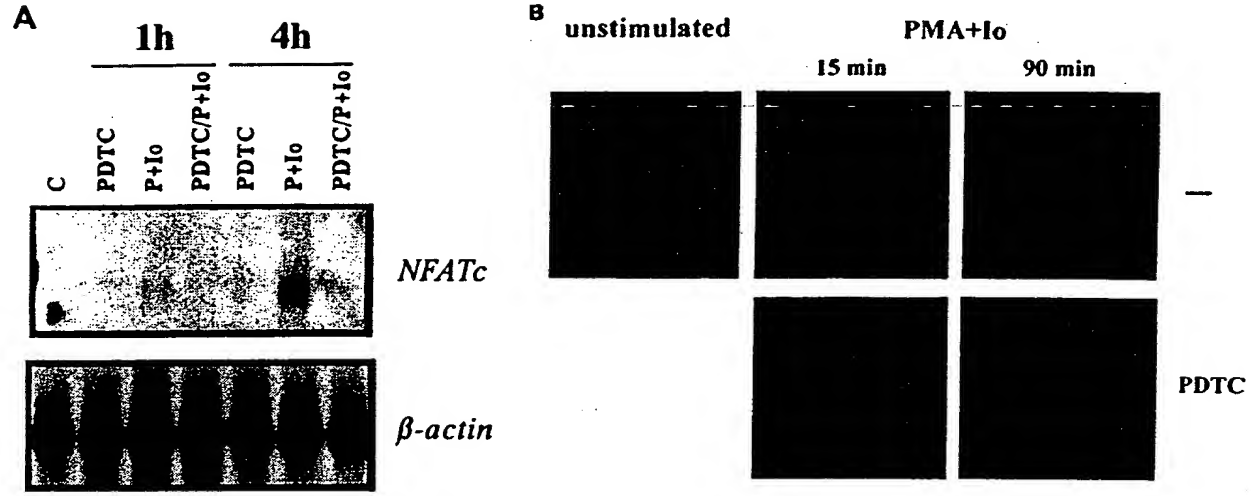

FIG. 10. Effect of PDTC on gene expression and nuclear translocation of NFATc in activated cells. (A) Poly(A)$^+$ mRNAs from Jurkat cells treated with 50 μM PDTC (PDTC) or 20 ng of PMA per ml plus 1 μM A23187 (P+Io) or pretreated for 2 h with 50 μM PDTC and then stimulated with 20 ng of PMA per ml plus 1 μM ionophore (PDTC/P+Io) for 1 and 4 h were isolated, separated by agarose gel electrophoresis, transferred onto a nitrocellulose membrane, and sequentially hybridized with NFATc and β-actin cDNA probes. C, control. (B) Staining of Jurkat cells transfected with HA-tagged NFATc pretreated or not with 50 μM PDTC for 2 h and further stimulated with 20 ng of PMA per ml plus 1 μM ionophore for 15 or 90 min. After incubation, cells were fixed and analyzed by immunofluorescence with anti-HA antibody. The subcellular localization of HA-tagged NFATc in resting and activated cells is shown in the upper panels, and that of PDTC-pretreated cells is represented in the lower panels.

displays a lower mobility than that of untreated cells that could be due to hyperphosphorylation of the transcription factor. Since we have shown that NFATp is directly phosphorylated by JNK and that PDTC exerts a potent and sustained activation of JNK in Jurkat cells (18), as well as in PBLs, it is conceivable that the sustained activation of JNK may be related to the rapid nuclear exit of NFATp. However, in vivo experiments will be required to elucidate whether JNK is indeed involved in regulating the shuttling of NFATp. Alternatively, a possible late effect of PDTC involving inhibition of calcineurin that would allow normal kinases to reverse NFATp dephosphorylation cannot be excluded. Independently of the mechanisms by which PDTC promotes the accelerated export of NFATp from the nucleus of activated cells, this rapid export may well account for its inhibitory effect on the transactivation of NFATp, and the identification of the cellular targets involved in this process may help the design of new strategies and drugs for immunosuppressive purposes. In this regard, it is important to note that maintenance of NFAT in the nucleus as a result of a sustained increase in Ca_i^{2+}, but not a transient increase, has been shown to be required to switch on IL-2 gene expression in activated Jurkat T cells (65).

The inhibition of activation both NF-κB and NFAT by DTCs suggests a potential use of these agents as immunosuppressive drugs. NFAT and NF-κB control the transcription of many cytokine genes and are involved in the response to proinflammatory stimuli in a number of cell types and in the T-cell activation process (3, 46). The effects of DTCs are not restricted to transformed T-cell lines; in primary human T lymphocytes, PDTC has been shown to inhibit cell proliferation and CD25 expression in response to costimulation with anti-CD28 and anti-CD2 MAbs (1), as well as the IL-2 secretion induced by costimulation with antibodies against TCR-CD3 and CD28 (30). As occurs with Jurkat T cells, in activated PBLs, DTCs inhibit cell surface expression of activation antigens and binding of NFAT and NF-κB to their cognate sequences in EMSAs. Based on their effects in vitro, it will be very important to analyze the potential of DTCs as immunosuppressive drugs in vivo. In fact, DDTC has already been used in human immunodeficiency virus-infected patients, and beneficial effects of the drug in delaying AIDS symptoms have been reported (47). However, several effects of DTCs observed in vitro must be taken into consideration when the use of these drugs in vivo is evaluated. First of all, DTCs activate AP-1 in a number of cell types. This effect has been involved in the upregulation of ICAM-1 expression in endothelial cells (40), and it is likely to mediate CD69 membrane expression and the activation of other AP-1-dependent genes. Although the overall effect of DTCs in cells analyzed seems to be inhibitory, AP-1-dependent gene activation could potentially interfere with the putative anti-inflammatory properties of these compounds. In addition, overdosage or long-term treatments with PDTC can desensitize cells to the inhibitory effect of the drug (53). Furthermore, although DTCs have been found to prevent apoptotic cell death (7, 13, 56), they have also been involved in triggering of apoptosis (42, 71). Although at the times and doses analyzed, DTCs do not affect the viability of Jurkat cells, we have observed that longer treatments with these agents result in loss of cell viability associated with a clear pattern of apoptosis. Since a role for NF-κB activation in the suppression of signals leading to cell death has been suggested (4, 6, 68, 69), it is possible that some genes whose expression is regulated by NF-κB may be involved in protective mechanisms against programmed cell death. Hence, these possible effects must be contemplated in cases in which in vivo protocols involving long-term treatments with DTCs are used. On the other hand, a potential benefit of DTCs versus other immunosuppressive drugs is related to DTC's ability to target other cells apart from those of the immune system. Thus, whereas CsA and FK 506 mediate in vivo immunosuppression preferentially through T lymphocytes, PDTC can also efficiently block cytokine production and the induction of the VCAM-1 and E-selectin expression in endothelial cells activated by proinflammatory stimuli (36, 41). Since VCAM-1 and ELAM-1 are adhesion molecules involved in leukocyte-endothelial cell interactions required for the recruitment and emigration of cells of the immune system

to inflamed tissues, it will be very important to determine whether such processes are affected by DTCs in vivo.

The ability of DTCs to inhibit the transcriptional activation of both NFAT and NF-κB in a variety of immunocompetent cells and the fact that these compounds have previously been used as pharmaceutical drugs support their potential use as immunosuppressants. While such use will require a careful and thorough analysis by evaluation of the overall effects of DTCs in vivo, DTCs have been shown to be useful probes for studying signaling pathways and regulation of transcription factors implicated in the T-cell activation process.

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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Eric OLSON *et al.*

Serial No.: 09/061,417

Filed: April 16, 1998

For: METHODS AND COMPOSITIONS FOR THERAPEUTIC INTERVENTION IN CARDIAC HYPERTROPHY

Group Art Unit: 1642

Examiner D. Minh Tam

Atty. Dkt. No.: MYOG:029US/SLH

CERTIFICATE OF MAILING
37 C.F.R. § 1.8

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July 24, 2002
Date

Steven L. Highlander

DECLARATION OF RICK GORCZYNSKI UNDER 37 C.F.R. §1.132

Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

I, Rick Gorczynski, do declare the following:

1. I am currently hold the position of Vice President, Research & Development at Myogen, Inc., licensee of the above-captioned application. My education and training includes an undergraduate degree in Biological Sciences from Cornell University and a Ph.D. in Cardiovascular Physiology from the University of Virginia, School of Medicine. I have worked since 1976 in the pharmaceutical industry, primarily in the cardiovascular drug discovery field. During my 25 years in the industry I have conducted and/or supervised

research directed at a variety of cardiovascular diseases including heart failure (acute and chronic), myocardial infarction, cardiac dysrhythmia, hypertension, renal disease, hyperlipidemias and thrombosis disorders. For the past 4 years I have been exclusively engaged in the discovery and validation of molecular drug targets for use in drug discovery in the filed of heart failure. I am intimately familiar with the use of transgenic mice in the field of cardiac research and heart failure. A copy of my *curriculum vitae* is attached.

2. I am also familiar with the level of skill of scientists working in the field of cardiology and molecular biology as of the priority date of the referenced application. I consider one of ordinary skill in the art in this field of study to have a Ph.D. in biochemistry, chemistry, molecular biology, pathology or other related field, or an M.D., with 1-3 years of post-graduate study.

3. I have reviewed the specification and pending claims 1, 4, and 9 for the above-referenced case. The specification refers to the use of transfected cells and transgenic mice where NF-AT3 is either overexpressed (transfected cells) or continuously activated (transgenics) as a model for hypertrophy studies that are then held out as subsequently relevant for human studies. More specifically the specification refers to NF-AT3 transgenes lacking one or more phosphorylation sites present in wild-type NF-AT3, NF-AT3 transgenes lacking all the phosphorylation sites of the wild-type protein, and NF-AT3 transgenes lacking amino acids 1-137 of the wild-type NF-AT3 protein.

4. The inventors' paradigm, as defined through the transgenic models, was that activated calcineurin would directly bind to and dephosphorylate cytoplasmic NF-AT3. The

dephosphorylated NF-AT3 would then translocate into the nucleus where it would act as a transcription factor along with GATA-4, leading to induction of hypertrophic genes. It has not yet been shown, as the examiner correctly points out, that NF-AT3 is constitutively active or in a more active state in the hearts of hypertrophic patients. Nonetheless, NF-AT3 presents itself as an attractive candidate for therapy in cardiac hypertrophy. To that end, the inventors have targeted NF-AT3 both directly and indirectly to inhibit the onset of the transcription of hypertrophic genes.

5. I have reviewed the enclosed article by Ritter *et al.*, entitled "Calcineurin in Human Heart Hypertrophy," *Circulation*, 105:2265-2269 (2002) which supports the inventors' claims relating to NF-AT3 as a therapeutic target. The Ritter *et al.* authors set out to validate the observations made in transgenic mouse models by studying enzymatic activity and protein expression in cardiac tissue from human patients suffering from hypertrophic obstructive cardiomyopathy. While the article focuses more on the data regarding calcineurin levels in the hypertrophic hearts, the researchers also studied NF-AT2 phosphorlyation levels in normal and hypertrophic heart tissue.

Ritter *et al.* showed that, in naturally hypertrophic tissues, NF-AT2 migrated at a higher rate on a 6% SDS gel, "compared with normal heart and identical to the NF-AT migration velocity of normal heart extracts treated with additional external calcineurin" (p. 2267), providing *in vivo* evidence from a human clinical setting of an altered NF-AT phosphorlyation state in hypertrophied myocardium. This shows that NF-AT2, is in a more active, dephosphorylated form in the human hypertrophic heart, and also strongly implicates a similar finding for NF-AT3. Moreover, it validates the present inventors' notion of targeting NF-AT3 therapeutically to combat hypertrophy by interfering with the

NF-AT3 transcriptional cascade, whether by direct blocking (as in binding a molecule to NF-AT3) or by indirect effects (targeting the purported NF-AT3/GATA-4 complex).

6. With regard to the issue of using transfected cells to elucidate the interaction between NF-AT3 and GATA4, I have reviewed the attached scientific publications entitled "The Zinc Finger-containing Transcription Factors GATA-4, -5, and –6" (*J. Biol. Chem.* 275:50, 38949-38952, 2000), and "Remodeling muscles with calcineurin," (*BioEssays* 22:510-519, 2000). Based on the results set forth in these papers, it is clear to me that a person of ordinary skill (as defined above) would recognize that the art currently accepts that NF-AT3 does indeed interact with GATA-4, although the has not been shown directly. Perhaps the best information on this point comes from the latter paper, a mini-review of the state of the art, published over a year and a half ago. This article states that "GATA-4 also physically interacts by way of the C-terminal zinc finger with nuclear factor of activated T-cells-c4 (NFAT)" (p. 38951; also see Molkentin, *Cell* 93, 215; and Morin, *EMBO J.*, 19, 2046).

In conclusion, though no single experiment demonstrates a physical interaction between GATA-4 and NF-AT3, the aforementioned authors, as well as those of ordinary skill in the art in the field of cardiac biology, believe that such an interaction takes place. Further, it is my opinion that targeting this interaction using the approaches set forth in the present specification, with which is am quite familiar, is a valid approach to the treatment of hypertrophy. In particular, the use of GATA-4 mimetics and other small molecules that would interrupt the NF-AT3/GATA-4 interaction would be expected to interfere with NF-AT3's stimulation of the hypertrophic genes, providing a benefit to the patient.

7. I hereby declare that all statements made of my own knowledge are true and all statements made on information are believed to be true and further that the statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both under § 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this application or any patent issued thereon.

July 3, 2007
Date

Richard Gorczynski
Rick Gorczynski, Ph.D.

CURRICULUM VITAE

RICHARD J. GORCZYNSKI, Ph.D.
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PROFESSIONAL EXPERIENCE

12/98-present
Vice President, Research and Development, Myogen Inc, Westminster, Colorado
Responsibilities

- Corporate Officer and member of the Myogen Executive Management Team
- Lead R&D activities focused on clinical development of one Phase II and one Phase III stage therapeutics for treatment of heart failure and related disorders; use of genomic, proteomic and biological techniques for identification of novel, disease-modifying compounds for reversal and prevention of heart failure; validation of molecular targets for drug discovery; identification of novel diagnostic markers for cardiac hypertrophy and heart failure
- Project Team Leader for Enoximone P.O. Development (until 1/00)
- Project Steering Committee member for BSF 208075
- Project Team Leader for Myosin Heavy Chain Project (collaboration between Myogen and a big pharma company)

Accomplishments

- In collaboration with Myogen scientific and medical advisory boards, established the company's Research Plan; established Target Validation techniques, HT Screening for the Company; built the R&D Group; managed external collaborations resulting in the identification of new technology for the Company.
- Project Plans for two Projects established: enoximone P.O. and Myosin Heavy Chain
- Planned and completed two successful meetings with the FDA Cardiorenal Division; resulted in agreement to proceed to Phase III with a heart failure therapeutic (enoximone); achieved alignment with the Agency on endpoints for four Phase II studies, product labelling language and scope of NDA.

6/98-11/98
Vice President, Research and Development and Boulder-Site Manager, Baxter, Hemoglobin Therapeutics Division, (Post-Baxter acquistion of Somatogen), Boulder, Colorado
Responsibilities

- Member of the Hemoglobin Therapeutics Division Management Team

- Lead Research and development activities at the Boulder-Site focused on the biological support of a Phase III-stage hemoglobin product candidate, DCLHb, research and development support for a Phase II-stage hemoglobin product candidate, rHb1.1 and research and development activities related to the discovery and advancement to clinical evaluation of a Second Generation hemoglobin product candidates
- Manage the Boulder-Site administratively, including facilities, safety, MIS and communications for R&D, Operations, Clinical, HR and Finance.

Accomplishments

- Defined new Boulder-Site organization in collaboration with the hemoglobin Therapeutics Division management team
- Identified several novel Second Generation hemoglobin product candidates

12/94 -6/98

Vice President, Research and Development, Somatogen, Inc., Boulder, CO

Responsibilities

- Corporate Officer and member of the Executive Management Group.
- Lead Research and Development activities at Somatogen; focused on 1) commercialization of lead product, Recombinant Human Hemoglobin (rHb 1.1) for oxygen-delivering and hematopoietic therapeutic indications 2) discovery and development of Second Generation recombinant hemoglobin products 3) development of non-hemoglobin technologies.
- Manage and coordinate Departments of Molecular Biology, Protein Engineering, Hemoglobin Research/Protein Chemistry, Pharmacology/Toxicology, Molecular Computation, Analytical Development, Purification Development, Formulation Development, and Fermentation Development (total of 65-70 people).

R&D Accomplishments

- In conjunction with the other Corporate Officers, positioned Somatogen for acquisition by Baxter Heathcare and prepared and presented the key technology summaries which lured Baxter to the table and eventually lead to an acquisition of Somatogen.
- Developed bioprocess for making clinical grade (GMP) rHb1.1 with successful scale-up demonstrating achievement of commercial expression and downstream yield targets; includes construction of host vector and strain, fermentation process, recovery and downstream purification system and associated analytical characterization; some of this was accomplished in collaboration with Eli Lilly and Co., our strategic corporate partner at that time.
- Advanced the understanding of hemoglobin biological effects including efficacy (oxygen delivery to tissue and potency), and safety related biologic effects; this work completed in support of commercial development of rHb1.1 and extended to the discovery of novel hemoglobin products.
- Initiated research on a promising new indication for rHb1.1: tumor radiation therapy sensitization.
- Initiated a drug-discovery project to identify new generation recombinant hemoglobin with enhanced therapeutic attributes; over 600 variant recombinant and

chemically modified/conjugated/cross-linked molecules constructed in three years; several lead molecules are undergoing advanced biological evaluation to ascertain suitability for human clinical testing. All have significantly improved properties.

- Three/four fold increases in rHb expression levels have been achieved (relative to commercial targets for rHb1.1).
- Completed several studies investigating the level of hematopoietic activity of rHb1.1 and other recombinant hemoglobins.
- Supervised preclinical discovery and development of a novel, in-licensed platelet substitute.

4/93 -12/94
Senior Director, Drug Discovery, Searle, Skokie, IL
Responsibilities

- Supervise the Cardiovascular Discovery Research Department (approximately 50 scientists; two sites: Skokie and St. Louis) with primary emphasis on atherosclerosis, thrombosis, arrhythmia, congestive heart failure and hyperlipidemia.
- Coordinate the process by which compounds from the Discovery Department are selected for, and transferred into, the Development Pipeline.
- Member R/D Executive Committee, Research Executive Committee and Development Executive Committee.
- Skokie Discovery Site Manager for facilities, safety and space administration.
- R/D liaison to the Corporate Licensing group.

Accomplishments

- Department Charter and Long Range Research Plan established.
- Five new Ph.D. hires in 1993 with backgrounds representing new directions in cardiovascular research (atherosclerosis/thrombosis/diabetes).
- Directed the design of a process by which Searle R/D will select Discovery stage compounds for formal Development; process consists of early toxicity, formulation, pharmacokinetic and chemical development studies of candidate molecules to optimize selection and the completion of critical analysis (development plan, marketing and financial) to support informal discussions on what to develop.
- Advanced new antiplatelet and antithrombotic agents into development (7/94); two antiplatelet compounds advanced to Phase III clinical development

8/89 - 4/93
Senior Director, Scientific and Product Affairs, Licensing/Business Development, Searle, Skokie, IL
Responsibilities

- Identification and follow-up of license and business development opportunities, with particular emphasis on Japan. Technical evaluation of all product license candidates.
- Manage process for full technical, medical and marketing review of candidates.
- Coordinate design of Development and Commercialization Plans for in-license candidates.
- Presentation of licensing opportunities to Searle top-management.

- Liaison between Licensing and Searle R/D.

Accomplishments:

- Two development collaborations initiated.
- One compound in-licensed (antidiabetic).

1/86 - 8/89

Director, Department of Cardiovascular Diseases Research, Searle, Skokie, IL

Responsibilities

- Supervise product discovery, chemical and biological research in the cardiovascular field with primary emphasis on hypertension, atherosclerosis, thrombosis and arrhythmia (staff: 45).

Accomplishments

- Four compounds into development (antihypertensive, and three antiplatelet agents).
- Two compounds in clinical study: antiarrhythmic and hypolipidemic.

9/85 - 1/86

Director, Biological Research Department, Searle, Skokie, IL

Responsibilities

- Supervised product discovery, biological research in four areas: cardiovascular, CNS, gastrointestinal and autocoid mediated diseases (staff: 80).
- Department was reorganized 1/86 following restructuring of all R/D after Monsanto takeover of Searle.

8/83 - 9/85

Section Head Pharmacology, American Critical Care (Division of Baxter Travenol Corp) (formerly Arnar-Stone Laboratories), McGaw Park, IL

Responsibilities

- Supervised drug discovery, biological research in the cardiovascular, ophthalmic and CNS areas including beta-blockers, positive inotropic asgents, antiarrhythmic agents, antiglaucoma agents and antiepileptic agents (staff: 13).

Accomplishments

- Five compounds into development (two beta-blockers, one antiglaucoma, one antiarrhythmic and one antiepileptic).
- Four IND's and one NDA (with approval).

9/80 - 8/83

Group Leader, American Critical Care (Division of American Hospital Supply Corp.) McGaw Park, IL,

Responsibilities

- Supervised drug discovery, biological research in the cardiovascular area (beta-blockers, cardiotonics and alpha blockers).

11/78 - 9/80
Senior Research Investigator, Arnar-Stone Laboratories (Division of American Hospital Supply Corp.), McGaw Park, IL,
Responsibilities

- Drug discovery in field of dopamine analogues and beta adrenergic receptor antagonists.

9/76 - 11/78
Research Investigator, Arnar-Stone Laboratories, McGaw Park, IL
Responsibilities

- Drug discovery in field of dopamine analogues and beta adrenergic receptor antagonists.

DRUG DEVELOPMENT EXPERIENCE

- Project Team Leader: enoximone P.O.; Phase II for treatment of ultra-advanced heart failure (myogen).
- Project Team Leader: Second Generation recombinant hemoglobin project (Somatogen).
- Designed the process used to select compounds for formal Development and Clinical Study (Searle).
- Liaison to Development Project Teams for all cardiovascular compounds accepted for development (Searle).
- Project Team Leader (American Critical Care).
- Coordinate design of Development and Commercialization Plans for in-license candidates.
- Development of an ultra-short acting beta-blocker - responsible for organizing and tracking development of a novel compound through all stages of preclinical development (raw material supplies, pharmacology, drug metabolism/pharmacokinetics, analytical assays, formulation, stability, etc.) and initial clinical trials.
- Member of three other project teams which are responsible for the development of a vasodilator, an antiarrhythmic agent and another ultra-short acting beta-blocker.

EDUCATION

1976 Ph.D., Physiology
University of Virginia
School of Medicine, Department of Physiology
Charlottesville, Virginia

Dissertation: The Microcirculatory Basis of Functional Hyperemia in Striated Muscle (University Microfilms #76-25012)
Thesis Advisor: Brian R. Duling, Ph.D., Professor of Physiology

1970 B.A., Biological Sciences
Cornell University
Ithaca, New York

TRAINING

1996 Somatogen: Performance Management System

1995 Somatogen: Project Management

1990 Searle: Introduction to Financial Analysis in Business (AMA)

1989 Searle: Introduction to Licensing (LES)

1989 Searle: Decision Making Skills: Consensus

1986 Searle: Interview Selection Skills

1986 Searle: Personnel Management System Training

1983 American Hospital Supply: Corporate Middle Management Course

1981 American Management Association: Project Management

1980 American Hospital Supply: Management Style and Effectiveness Training

AWARDS

American Critical Care President's Award for Scientific and Technical Excellence - 1979

Runner-up for American Critical Care President's Award for Scientific and Technical Excellence - 1978

PROFESSIONAL ACTIVITIES

Member, Editorial Board of the Journal of Cardiovascular Pharmacology - 1984 to 1994

Ad Hoc Reviewer for Microvascular Research, the American Journal of Physiology and the Journal of Pharmacology and Experimental Therapeutics, Blood

SOCIETIES

International Society for Artificial Cells, Blood Substitutes and Immobilization Biotechnology (Scientific Steering Committee)
American Society for Pharmacology and Experimental Therapeutics
American Association for Advancement of Science
International Society for Heart Research
Licensing Executives Society
American Heart Association

PATENTS

Novel therapeutic and diagnostic agents for treatment of heart failure. Applied May, 1999.

Epoxy-Steroidal Aldosterone Antagonist and Angiotensin II Antagonist Combination Therapy for Treatment of Congestive Heart Failure. WO96/40257

SEMINARS

1. Department of Physiology, University of Virginia, Fall 1976. "The microcirculatory basis of functional hyperemia in striated muscle".

2. Department of Physiology, Medical College of Wisconsin, Fall 1977. "The microcirculatory basis of functional hyperemia in hamster striated muscle".

3. Cardiovascular Discussion Group, Skokie, IL, Fall 1982. "Mechanisms of inotropic selectivity".

4. Esmolol Symposium, Spring 1985. "Basic pharmacology of esmolol".

5. Kureha Chemical Industry, Tokyo, Fall 1989. "Platelet GPIIb/IIIa: a new target for discovery of novel antiplatelet agents".

6. University of Virginia, Graduate Study Colloquium, Winter, 1994. "Job Opportunities in the Pharmaceutical Industry."

7. IBC Conference Blood Substitute, 1996. "Measurement of the Efficacy of Hemoglobin-based Oxygen Carriers

8. International Symposium on Intensive Care and Emergency Medicine, Brussels, 1997. Preclinical update on rHb1.1

9. Tokyo Blood Substitutes Conference, 1997. "Comparison of Optro with Whole Blood using 31 P-NMR Spectroscopy"

PUBLICATIONS

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ABSTRACTS

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12. Sum, C.Y., Lee, R.J., Yacobi, A. and Gorczynski, R.J.: Pharmacodynamics of ASL-8052-an ultra-short acting beta blocker (USABB) in anesthetized dogs. Federation Proc. 42, 1139, 1983

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Antisense Has Growing Pains

Efforts to develop antisense compounds as therapies for cancer, AIDS, and other diseases have encountered some unexpected questions about how the drugs really work

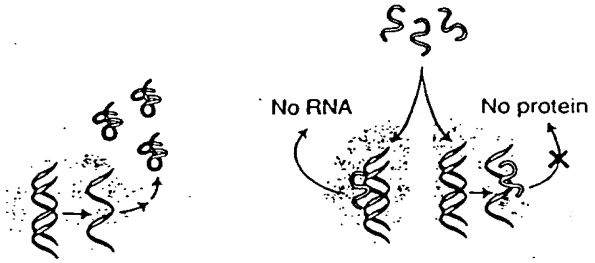
When *Science* named the gene-blocking technique known as antisense technology runner-up for its 1992 "Molecule of the Year," the accolade seemed well deserved. At the time, the technology appeared to offer a promising way to turn specific genes on or off at will. And that had made it potentially a powerful tool for uses ranging from fundamental molecular biology to the development of pharmaceuticals. Indeed, firms, both new and established, were rushing to exploit the technology to produce novel, rationally designed drugs for treating conditions ranging from genetic diseases to viral infections, including AIDS, and even cancer.

But during the past few years, the technique has run into unforeseen problems, and some of that early gloss has begun to wear off. Although several clinical trials have already begun, and there have been some promising results, researchers have encountered difficulties in getting antisense drugs—usually short pieces of DNA (called oligonucleotides) that have been designed to recognize and bind to specific genes—into target tissues. And potentially toxic side effects, including decreased blood clotting and cardiovascular problems such as increased blood pressure and decreased heart rate, have shown up in animal studies that have served as the basis for early human trials. But the biggest concern is that antisense compounds simply don't work the way researchers once thought they did.

"The assumption is that we are designing oligonucleotides that don't interact with anything besides [their targets]," says Cy Crein, an assistant professor of medicine and pharmacology at Columbia University's College of Physicians and Surgeons in New York City. "Many people are worried that a lot of the positive effects reported are not just antisense but other nonantisense mechanisms as well."

This uncertainty about what antisense drugs are doing inside the body has caused some experts in the field to argue that clinical trials have begun far too soon. "It is too early to take these things to human beings ... when we don't even know how they are working in a test tube," contends Ramasamy Narayanan, who studies antisense drugs at Hoffmann-La Roche Inc. in New Jersey, but is not involved in any of the trials.

Others argue that even if the basic researchers haven't yet worked out the drugs' mechanisms of action, clinical trials are justified as long as the compounds show signs of efficacy. "As a clinician, what matters to me is if the drug works," says Jeffrey Holt, a pathologist at Vanderbilt University in Nashville, Tennessee, who is currently trying to use antisense DNA to fight advanced-stage breast cancer. "In medicine, people give drugs that we don't know the mechanism for." As one example, he cites aspirin, whose mode of action was not understood until relatively recently, even though it's been widely used for a century.



Holding on. In an untreated cell *(left)*, a gene's double-stranded DNA is transcribed into RNA, which then makes the protein *(green)*. Antisense drugs *(yellow)* are supposed to block this, by binding to the gene *(near right)* or the RNA *(far right)*. But do they?

Early promise

One reason antisense technology looked like the answer to drug designers' prayers is that it seemed to be simple and straightforward. During the first step of protein synthesis, in which genes are copied into RNA, only one strand of the double-helical DNA is so transcribed. The original idea, developed in the late 1970s and first published by Harvard Medical School researcher Paul Zamecnik, was to create a second RNA or DNA with a particular gene's complementary sequence—the so-called antisense molecule—that could recognize and bind to the RNA. This was supposed to prevent the RNA from manufacturing its protein, either directly or by causing it to be broken down by RNA-cutting enzymes. In the years since then, the technology has undergone several modifications, however.

To try and produce new drugs, researchers chemically string together a sequence of about 20 DNA bases—the oligonucleotide—that mirrors a short stretch of the gene scientists want to block. These may act either by binding to the RNAs, as the longer molecules do, or by binding directly to a target gene, thereby preventing it from being transcribed into RNA in the first place. (This latter approach is sometimes called "triplex" technology because a third DNA strand is being added to the two already in the DNA double helix.)

But however they work, such short oligonucleotides are much easier to synthesize than long antisense RNAs or DNAs. Researchers also made them more resistant to the many enzymes that break down nucleic acids by replacing a critical oxygen atom in each nucleotide building block with a sulfur atom. That's an important plus for a drug that has to be administered to a live human being, as it helps ensure that the drug will last long enough to do its job.

These modifications seemed to put drug designers on the right track: In initial tests with cultured cells, the sulfur-modified oligonucleotides, called phosphorothioates, appeared to work. For example, a team at Hybridon Inc., a biotech firm in Worcester, Massachusetts, found that one of their phosphorothioates, which they called GEM91, blocks replication of the AIDS virus, HIV-1, by targeting a viral life cycle gene called *gag*. "The antisense compound can suppress viral activity in vitro by up to 100%, depending on the concentration we use," says Sudhir Agrawal, vice president of drug discovery and chief scientific officer of the company. Other researchers also had early success in blocking reproduction of HIV-1 and other viruses with the sulfur-modified antisense constructs.

The successes quickly spurred the start-up of several biotech enterprises, such as Gilead Sciences Inc., an 8-year-old biotechnology company based in Foster City, California. "When we began, we said, 'Obviously from the literature, the technology works,' " recalls Richard Wagner, a molecular biologist at the company. "We thought that all we needed to do was bring in a few chemists and we were going to be rich."

But shortly after setting up shop, Gilead researchers realized it wouldn't be that simple. They quickly found that antisense

SOME CURRENT U.S. ANTISENSE CLINICAL TRIALS

Company	Disease	Rationale	Number of Patients
Isis Pharmaceuticals	CMV retinitis in AIDS patients	Block CMV reproduction	200+
Isis Pharmaceuticals	Genital warts	Block human papilloma-virus reproduction	70+
Isis Pharmaceuticals	Kidney transplant rejection	Block immune cell activities	20 to 40
Isis Pharmaceuticals	Rheumatoid arthritis and other autoimmune diseases	Block immune cell activities	20 to 40 per disease
Lynx Therapeutics	Chronic myelogenous leukemia	Block cancer gene activities	50+
Hybridon	AIDS	Block HIV reproduction	125

compounds applied to a strain of human blood cells did not even get into the nucleus, the site of their RNA or DNA targets, Wagner explains. To get around that problem, they were forced to inject the compounds directly into the cells, a technique that works well in laboratories but cannot be applied to patients.

They did get some encouraging results, though: When they performed the injections, Gilead workers found that compounds directed at the *rev* or *gag* genes located in HIV-1 inhibited viral replication in the cells. In other experiments, antisense oligonucleotides targeted to the *c-myc* gene of blood cells from leukemic patients shut down cancer cell proliferation. But in both sets of experiments, yet another glitch cropped up.

To their surprise, researchers found that oligonucleotides they were using as controls, which couldn't recognize the *rev*, *gag*, or *c-myc* genes, either shut down virus replication or blocked cell proliferation almost as effectively as the ones they were testing as drugs. "While we could repeat many of the biological effects caused in cell culture, in every case our controls would show the same response," Wagner notes. "When we went back to the original papers, we found that often these controls were missing."

At first, Gilead researchers kept their concerns quiet. "There were a significant number of people claiming that these things worked," Wagner explains. "We really didn't want to go public with our negative results until we were sure that we weren't doing something wrong in our system." By the early 1990s, however, other researchers were echoing Wagner's concerns.

One example comes from Arthur Krieg of the University of Iowa, Iowa City, and his colleagues, who were attempting to develop antisense compounds that could be used to treat autoimmune diseases, such as rheumatoid arthritis, in which the immune system begins attacking the body's own tissues. "The B cells in autoimmune disease are hyperactive," Krieg explains. "We were trying to identify the genes responsible and shut them down."

When the researchers tried to inhibit B cells in culture with antisense DNA, however, the molecules turned B cell function up instead of down. That result was a mixed blessing, because it suggested that while the compounds tested would not be useful for treating autoimmune diseases, they might help buttress immune cell function in AIDS patients. But the Iowa team encountered an anomaly in their system similar to the one the Gilead workers had previously found. "Later, we got concerned as a number of controls also turned out to be B cell activators as well," Krieg recalls.

The immunologist, who says he worked "full time" to figure out what was causing this, came up with a solution earlier this year. In a paper published in the 6 April issue of *Nature*, Krieg and his colleagues reported evidence suggesting that antisense oligonucleotides mimic bacterial DNA in triggering a potent response by mammalian immune cells. They based this conclusion on experiments in which they showed that DNA fragments containing the two-base sequence CpG (where C stands for the nucleotide base cytosine, the G for guanine, and p for phosphate) activate mammalian B cells and natural killer cells in culture.

This only takes place, however, when the CpG motif lacks methyl groups. Because such sequences are common in bacterial DNA, but not in mammalian DNA, where most nucleotides have an attached methyl group, the immune response may be a way of defending against bacterial infections, Krieg suggests. The finding applies to antisense technology because antisense manufacturers don't usually add methyl groups to their synthetic oligonucleotides. Thus, mammalian immune systems that encounter such compounds with the CpG motif may be tricked into thinking they have been invaded by bacterial aliens and consequently spring into action.

Krieg suggests that this response could be useful clinically, but he says researchers need to be aware that the drugs are working directly on the immune system, rather than, say, targeting the AIDS virus itself. "I firmly convinced that synthetic oligonucleotides, like the ones in clinical trials will make useful drugs for human bei Krieg says. "But I don't think they are v ing through true antisense mechanisms

Side effects in animals

Besides not always working by "true sense mechanisms," the synthetic o nucleotides have also caused side effec experimental animals. When administ by one-time injection in high dose monkeys, for example, several phosph thioate drugs were lethal to some of the mals, for reasons that are not yet underst In others, the oligonucleotides caused a t sient decrease in the total number of kinds of white blood cells as well as chai in blood pressure and heart rate, accordin Hybridon's Agrawal. In addition, phosp rothioates have been found by Hybridon Isis researchers to accumulate in the li kidneys, and bone marrow of animals, though the long-term effects of this dep tion are not clear.

Some of these effects may be explainec the drugs' propensity to bind to proteins, Columbia's Stein. At a recent meeting the "Art of Antisense,"* molecular phari cologist Stein presented some of his tea findings on why the compounds often dc make it to the nucleus. They've found t they end up instead in the endosomes, sn membrane-bound vesicles in the cytopla: This apparently occurs because the oli nucleotides tend to bind to proteins, wh are themselves incorporated in the en somes. "Many cell types protect themsel by sequestering oligos in intracellular co partments," Stein says, but this could a contribute to the deposition of the drugs liver and kidney.

In addition to getting entangled by p teins inside cells, the Columbia researcl found that many synthetic oligonucleotid because of their highly negative charge, hung up on proteins outside cells as w Among these are growth factors and cell choring proteins such as fibronectin a laminin. The result is that antisense co pounds block cell migration and adhesion underlying tissue in vitro—an effect tl may interfere with wound healing and ar rial wall repair in living animals, Stein sa

Hybridon's Agrawal maintains, howev that the cardiovascular and other effects se in animals can be minimized in patients using low doses of the compounds and a ministering them gradually by continuc intravenous injection. That seems to borne out by the early results of Hybridor

* The meeting, which was sponsored by *Nati Medicine*, was held in New Orleans on 21 an 22 September.

clinical trial of GEM91 in AIDS patients, he told participants in the antisense meeting. Agrawal also reported that patients getting the higher doses are showing signs of clinical improvement in that their viral counts drop a few days after the treatments, although it is far too soon to tell whether this translates into improved survival. To Agrawal, it doesn't matter how the drugs work, if they end up helping AIDS patients. "Despite all the other properties [in addition to actual gene targeting], we feel that if we find an antisense effect ... then we have a new drug," Agrawal says.

Looking to the future

Agrawal is not the only one who hasn't lost faith in the technology. Biotechnology representatives argue that the problems turning up with antisense oligonucleotides are common in drug development, especially when untested, new technologies are being explored. "Every new technology starts at the bottom, in essence, getting your foot in the door," says Gerald Zon, vice president of medicinal chemistry at Lynx Therapeutics Inc., a biotech company in Hayward, California. He notes that every new drug has negative effects that must be weighed against clinical benefits. The answer, he says, is to design better second- and third-generation drugs in order to boost drug efficacy while, at the same time, minimizing unwanted side effects.

Indeed, researchers at companies such as Hybridon, Isis, and Gilead say they are applying the lessons they are learning from the animal studies and early clinical trials to try to come up with better and less toxic compounds. The options they are exploring include modifying the structures of oligonucleotides so that they bind less readily to proteins or more readily to their target genes. All three companies are also generating fat-soluble delivery molecules called cationic liposomes. The researchers hope these lipid-loving shuttles will help antisense compounds break through cellular barriers that prevent entrance into the nucleus.

These new compounds and delivery systems carry no guarantees that they will be any better than the phosphorothioates used in the current clinical trials. But even some of the critics, such as Stein, agree the field still holds great promise, as long as the researchers recognize that antisense drugs don't always work the way they are supposed to. "My guess is that we will find that the current generation of phosphorothioates are extremely active biological molecules," Stein concludes, "and that they work by many mechanisms, of which antisense is one. The truth is that we'll have to wait and see. None of us really knows what is going to come out of it."

–Trisha Gura

Trisha Gura is a reporter on leave from the Chicago Tribune.

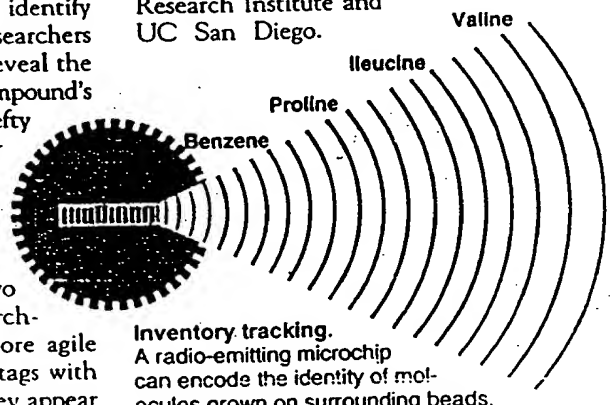
CHEMISTRY

Radio Tags Speed Compound Synthesis

Like aging computers, it doesn't take long for scientific techniques to seem slow and cumbersome. Take combinatorial chemistry. When it was introduced a few years ago, it was the supercomputer of chemical synthesis. The technique allows chemists to quickly paste together several different chemical building blocks into millions of combinations, in hopes that one will prove to be a new drug or a useful material. To identify each one of the new compounds, researchers typically affix chemical tags that reveal the unique arrangement of each compound's components. But these tags carry a hefty price: Their use doubles the number of chemical steps—and the time—involved in the assembly process, and their fragility prevents the synthesis of some compounds.

In the past 2 weeks, however, two separate groups of California researchers have unveiled a faster and more agile model. By replacing chemical ID tags with tiny radio-emitting microchips, they appear to have overcome both of the problems inherent in the old one. "The upshot is that it makes the whole process of drug discovery more efficient," says Rob Armstrong, a chemist at the University of California (UC), Los Angeles, who led one of the research groups, which includes scientists from Ontogen Corp. in Carlsbad, California. "This has the potential to be a significant advance in simplifying the encoding process," adds Michael Pavia, who heads combinatorial research at Sphinx Pharmaceuticals in Cambridge, Massachusetts. The technique not only saves time, says Pavia, "it gives you a wider range of chemical diversity to select from in building your new molecules."

Armstrong's group presented its findings at last week's meeting of the Western Biotech Conference in San Diego, as did the second team, led by Michael Nova at IRORI Quantum Microchemistry in La Jolla, California, and K. C. Nicolaou, who holds dual appointments at the La Jolla–based Scripps Research Institute and UC San Diego.



Inventory tracking. A radio-emitting microchip can encode the identity of molecules grown on surrounding beads.

The IRORI group was, however, the first in print, with a paper in the 15 October issue of *Angewandte Chemie*.

Both techniques add considerable power to combinatorial chemistry, which already made traditional synthetic chemistry look like an old IBM punch card. Traditionally, novel compounds are synthesized one at a time, but combinatorial chemists create huge numbers in a single process by assembling a few chemical building blocks—each of which has a corresponding ID tag, such as a short nucleotide sequence—in all possible combinations. Chemists need these tags to decipher the makeup of compounds that show promise in an assay, such as the ability to kill cancer cells (*Science*, 3 June 1994, p. 1399).

But because a tag has to be added with each building block, assembling a 10-component molecule actually involves at least 20 time-consuming chemical steps. And the technique runs into trouble when creating small organic molecules, which constitute most of today's drugs. Some of the synthetic reactions involve potent reagents, such as hydrofluoric acid, which can rip ID tags apart.

The new microchip tags appear to solve both these problems at once. A chip, which emits a binary code, is inserted into a mesh capsule loaded with polymer beads—the "seeds" to which combinatorial building blocks are added by dunking the capsule in a series of beakers. In the Ontogen approach, a nearby radio scanner registers both the identity of a capsule and the contents of each beaker it enters. These data are uploaded to a computer that keeps track of the order of building blocks in the growing molecule. In the IRORI approach, the information is stored on the microchip itself, using a transmitter that writes the information to the chip. The information is uploaded to the computer only when the assembly run is complete.

By eliminating the chemical tags, both approaches do away with half the synthetic steps involved, yet end up with an instantly available computer record of the precise structure of the compounds in each capsule. Moreover, says Nicolaou, "now you are free to use any chemistry you want to build your molecules." Speed and flexibility—for chemists, it's a winning combination.

–Robert F. Service

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COMMENTARY

Antisense Oligonucleotides: Is the Glass Half Full or Half Empty?

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ABSTRACT. Antisense oligonucleotides are widely used as tools to explore the pharmacological effects of inhibiting expression of a selected gene product. In addition, they are being investigated as therapeutic agents for the treatment of viral infections, cancers, and inflammatory disorders. Proof that the pharmacological effects produced by the oligonucleotides are attributable to an antisense mechanism of action requires careful experimentation. Central to this problem is the finding that oligonucleotides are capable of interacting with and modulating function of specific proteins in both a sequence-independent and -dependent manner. Despite these undesired interactions, it has been possible to demonstrate that oligonucleotides are capable of binding to a specific RNA in cultured cells, or within tissues, resulting in selective reduction of the targeted gene product and pharmacological activity. In general, these oligonucleotides were identified after a selection process in which multiple oligonucleotides targeting different regions on the RNA were evaluated for direct inhibition of targeted gene product, resulting in the identification of a potent and selective oligonucleotide. Similar to other drug-receptor interactions, selection of the most potent inhibitor results in an increase in the signal-to-noise ratio, yielding increased confidence that activity observed is the result of a desired effect of the inhibitor. With careful selection, proper controls, and careful dose-response curves it is possible to utilize antisense oligonucleotides as effective research tools and potentially as therapeutic agents. BIOCHEM PHARMACOL 55;1:9–19, 1998.

KEY WORDS. antisense oligonucleotides; pharmacology; toxicology; pharmacokinetics; nucleic acids

Solving the genetic code and identification of methods for manipulating genetic information in cells have provided the framework for the current revolution in biomedical sciences. One of the most direct applications of this information is the design of short oligonucleotides (12–25 bases in length) that hybridize to a specific mRNA by Watson-Crick base pairing rules. Upon binding to the mRNA in the cell, the antisense oligonucleotide prevents expression of a protein product encoded by the targeted RNA. Antisense oligonucleotides have the potential to be a truly rational approach for drug design in that the rules for binding have been well characterized. Conceptually, such an approach is very attractive, in that all that is needed to develop an inhibitor is the sequence of the RNA of interest and the ability to synthesize the oligonucleotides. In fact, numerous studies have been published claiming inhibition of a wide variety of gene products with antisense oligonucleotides both in cell culture based experiments and *in vivo* [1–3]. However, it has become increasingly clear that like any other scientific endeavor, practicing antisense technology requires careful experimental design and interpretation of the results, in that some of these published studies probably identified pharmacological activity of the oligonucleotide which cannot be attributed to an antisense effect. Because of these findings, there has been some criticism of the technology in the scientific literature [4–6]. Although some of these criticisms have validity, it is important to put them in perspective. The ultimate questions are (1) whether the mechanism of action for antisense drugs can be well enough defined to allow their use as research tools, and (2) will antisense oligonucleotides be useful therapeutic agents. I will attempt to answer these two questions in this commentary and, in so doing, discuss what some of the early perceived issues were for antisense technologies, which of these have proven to be valid issues, where some of the pitfalls have occurred in using antisense oligonucleotides, and finally what the prospects are for the future of the technology. I will not attempt to perform a comprehensive review of the topics, but rather use this as a forum to stimulate additional discussion and focus attention on what I feel are important issues the technology faces in 1997.

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ANTISENSE OLIGONUCLEOTIDES: PERCEIVED AND REAL ISSUES

Pharmacokinetics

CAN STABLE OLIGONUCLEOTIDE ANALOGS BE IDENTIFIED? Protection of the oligonucleotide from nuclease degradation has been one of the major accomplishments, resulting

from the application of medicinal chemistry to antisense technology. Chemical modifications have been identified that decrease degradation of the oligonucleotides, some even before the application of oligonucleotides for regulation of gene expression was fully appreciated. Some of the early modifications include phosphorothioate linkages [7, 8], phosphotriesters [9], methylphosphonate linkages [10, 11], and α-oligonucleotides [12]. More recently additional modifications have been identified that exhibit nuclease resistance, such as phosphorodithioate [13], additional 2′-modifications [14–17], 2′-5′ linkages [18], MMI† [19], formacetal [20], N3′ → P5′ phosphoramidate [21], amide 3 [22], and PNA [23].

Of these modifications, phosphorothioate oligodeoxynucleotides are the most widely used and currently account for all but one of the antisense oligonucleotides currently in the clinic. The stability of phosphorothioate oligodeoxynucleotides varies depending on the cell line or tissue investigated. In cell-based assays, phosphorothioate oligodeoxynucleotides have been reported to reduce expression of targeted mRNA for 24–48 hr [17, 24, 25]. If longer suppression of target RNA is desired, then cells either should be retreated with the oligonucleotide or additionally modified oligonucleotides can be used such as 2′-alkoxy [15, 25] or modifications in which the phosphorus has been replaced. *In vivo*, phosphorothioate oligodeoxynucleotides are metabolized in both serum and tissues [26–28]. The tissue half-life for intact drug is somewhat variable depending upon the sequence of the oligonucleotide and the tissue being studied [26–28]. More nuclease-resistant modifications would be expected to exhibit tissue half-lives significantly greater than these values [26, 29, 30]. With these modifications, dosing frequency could be reduced for better patient convenience. Thus, with a large number of modified oligonucleotides to select from, the degree of nuclease resistance desired in an oligonucleotide can be tailored to meet specific needs.

DO OLIGONUCLEOTIDES GET INTO CELLS? Alternatively, will oligonucleotides reach their intracellular target in sufficient amounts to inhibit expression of the targeted RNA? More importantly, are the pharmacokinetics of the oligonucleotide favorable enough to allow use for *in vivo* applications? Like most other drugs, the mechanisms by which oligonucleotides and their analogs interact with plasma components, distribute to tissues, and accumulate within cells are poorly understood. The pharmacokinetics of phosphorothioate oligodeoxynucleotides have been well described in terms of general plasma kinetics and tissue distribution [28, 31–34]. Phosphorothioate oligodeoxynucleotides are rapidly distributed out of plasma to most peripheral tissues, with liver and kidney accumulating the highest concentrations of oligonucleotide. Significant concentrations are also obtained in other tissues such as intestine, spleen, skin, and bone marrow. Thus, phosphorothioate oligodeoxynucleotides readily accumulate in a broad range of tissues.

Information on where within tissues the compounds accumulate is beginning to appear [35–37]. These studies suggest that at early times, up to 2 hr, the oligonucleotide is both associated with extracellular components and localized intracellularly [37]. At later times the bulk of the oligonucleotide is localized inside cells within the tissues. As expected, the oligonucleotides do not distribute uniformly within a tissue but accumulate within certain cell populations. Best characterized is the kidney, in which the oligonucleotide accumulates within epithelial cells of the proximal convoluted tubule [33, 37, 38]. Within other tissues, the oligonucleotide clearly localizes within specific cell populations; however, identification of the cell populations is more difficult.

Does enough oligonucleotide accumulate within cells to inhibit expression of targeted gene products? A very rough estimate for mRNA concentration in a cell that expresses 10,000 copies of a target mRNA (a relatively abundant mRNA) would be 20 nM. At a pharmacologically relevant dose of 3–6 mg/kg, the bulk concentration of oligonucleotide in kidney and liver is approximately 5–15 and 1–5 μM, respectively. Concentrations in other tissues range from less than 0.05 μM (brain) to 1 μM. One should next recognize that the oligonucleotide is accumulating within certain cell types within tissues, which could increase the actual cellular concentration at least 10-fold for some cell types. These concentrations, in theory, would be far in excess of target mRNA concentrations and therefore should inhibit targeted gene expression within tissues if they have access to the mRNA. This latter point is the one issue that has generated the most controversy, i.e. do oligonucleotides that are internalized in cells have access to the target RNA? Unfortunately, the answer to this question is complicated, but appears to be affirmative for some cell types within tissues.

Numerous investigators have characterized oligonucleotide internalization in mammalian cells. These studies, not unexpectedly, have yielded conflicting results in that different methods were used for evaluation of oligonucleotide internalization, different cell lines were used, and different oligonucleotides were used. The one consistent conclusion that can be drawn from these studies is that all mammalian cells investigated are capable of internalizing phosphorothioate oligodeoxynucleotides by an active process. The intracellular fate of the oligonucleotide is controversial. Some studies suggest that oligonucleotides are internalized by a "receptor" mediated or adsorptive endocytosis pathway in which the oligonucleotide is retained within membrane-bound intracellular vesicles [39–42], while other studies suggest that the oligonucleotide either uses alternative methods for gaining entry into cells or escapes from the cytoplasmic vesicles [43–45]. Our studies would suggest that there are multiple competing mechanisms by which

† *Abbreviations:* CMV, cytomegalovirus; ICAM-1, intercellular adhesion molecule-1; MMI, methylene (methylimino); NK cells, natural killer cells; and PNA, peptide nucleic acid.

oligonucleotides are internalized in the cells [46]. For the majority of cultured cell lines that we have investigated, we observed localization of fluorescently labeled oligonucleotides in cytoplasmic structures and failed to observe specific antisense effects in the absence of a facilitator such as cationic lipids. However, we and others have demonstrated that some specific cell types accumulate phosphorothioate oligonucleotides in the cell nucleus without use of cationic lipids or other transfection methods [44, 45]. We have also observed that *in vivo* administered oligonucleotide exhibits both patterns of distribution within specific cell types in tissues [37]. These data are supported by pharmacological studies in which it is possible to demonstrate reductions in target RNA or protein expression in tissues following systemic administration of the antisense oligonucleotide [47–56]. It is unlikely that there will be a single mechanism by which oligonucleotides become internalized in cells. Furthermore, data would suggest that behavior of oligonucleotides in cell culture does not predict their behavior *in vivo*.

Pharmacology

MECHANISM OF ACTION. What is the mechanism by which antisense oligonucleotides inhibit expression of the targeted gene product? Is occupancy of the RNA by the oligonucleotide sufficient or does the oligonucleotide need to exploit a catalytic activity such as RNase H? There are multiple theoretical mechanisms by which oligonucleotides can be used to regulate expression of target genes [57–59]. Perhaps the most widely used mechanism is cleavage of the targeted RNA by RNase H. Although it has not been unequivocally demonstrated that reduction or cleavage of the targeted RNA in cells is mediated by RNase H, there is a great deal of evidence to support such a conclusion, including direct demonstration of a reduction in target mRNA, demonstration of appropriate cleavage products [60–62], and use of modified oligonucleotides that do not support RNase H activity [24, 63, 64]. In addition to RNase H, mammalian cells express a variety of other RNases, which could be exploited to selectively inhibit expression of a targeted gene. Examples include RNase L [65] and double-stranded RNases.

There are also steric mechanisms by which oligonucleotides can prevent expression. Translation arrest, in which the oligonucleotide binds to the target mRNA and blocks movement of the ribosome and subsequently translation of the mRNA, is one of the most cited mechanisms of action. Several studies have been able to document that oligonucleotides are capable of inhibiting translation in an *in vitro* translation assay, including experiments by Ochoa and colleagues in 1961 [66]. However, it is more difficult to prove this as a mechanism in cell-based assays. Reduction in targeted protein by an oligonucleotide but no reduction in mRNA has been used as evidence for a translation arrest mechanism. Alternatively, demonstration of a selective reduction in target protein by modified oligonucleotides that do not support RNase H has also been used as evidence for a translation arrest mechanism. However, in both cases other mechanisms of action could account for these observations. Therefore, evidence directly demonstrating a translation arrest mechanism in cell culture or *in vivo* is circumstantial.

A somewhat related mechanism is prevention of ribosome assemble on the mRNA. Baker *et al.* [67] utilized uniformly 2′-modified oligonucleotides, which do not support RNase H activity, to target the 5′-terminus of ICAM-1 RNA [67]. These oligonucleotides very effectively inhibited ICAM-1 protein expression by markedly changing the polysome profile of ICAM-1 mRNA, shifting it from a higher molecular weight polysome pool to a lower molecular weight pool.

Regulating pre-mRNA maturation is also a potential mechanism by which oligonucleotides may inhibit or alter gene expression by sterically blocking recognition of the RNA. Kole and colleagues [68] have performed a series of studies demonstrating that uniformly modified 2′-O-methyl phosphorothioate oligonucleotides, which do not support RNase H, can alter the splicing of a thalassemic β-globin mRNA in mammalian cells. The oligonucleotide was used to mask a splice site so that an alternative site was utilized. Hodges and Crooke [69] have also reported similar findings in which they demonstrated that 2′-O-methyl oligonucleotides are capable of selectively blocking splicing of an adenovirus transcript.

These results demonstrate that there are multiple antisense mechanisms by which oligonucleotides can inhibit expression of genes. It is possible to utilize endogenous enzymes in cells to provide catalytic activity as a mechanism, or sterically blocking a critical regulatory element can be a very effective mechanism for inhibiting expression. The mechanism of action is dependent in part on where on the RNA the oligonucleotide hybridizes as well as the type of oligonucleotide chemistry used.

DO OLIGONUCLEOTIDES PRODUCE PHARMACOLOGICAL EFFECTS BY AN ANTISENSE MECHANISM OF ACTION, IN VITRO AND IN VIVO? There are multiple published experiments that demonstrate direct reduction in targeted gene expression by oligonucleotides, in which it is difficult to conclude that they are working by any other mechanism [as examples, see Refs. 24, 47, 63, 64, and 70–79]. In general, these studies had in common several of the following experiments to provide evidence that the oligonucleotide was, in fact, working by an antisense mechanism of action. (1) In these experiments, the oligonucleotides were selected for potency following a screen in which the expression of the target gene was directly analyzed, rather than indirect assays. By selecting for more potent oligonucleotides the signal-to-noise ratio is increased, allowing for characterization of pharmacological effects due to inhibition of the targeted gene product. This selection process at this time is rather empirical and should be conducted regardless of the type of chemistry. (2) These studies demonstrate reduction in

either the mRNA which codes for the protein of interest or the protein itself. (3) Control oligonucleotides were examined for activity, in some cases multiple controls. (4) In some experiments rank order potency comparisons were performed between *in vitro* and *in vivo* studies. (5) The studies demonstrate that the effects of the antisense oligonucleotide are specific for the targeted gene, in that related gene products were not inhibited. (6) Dose–response curves were performed with the oligonucleotide rather than single points. (7) The observed pharmacological activity was consistent with what was known about the biology of the target. (8) In most of the cell-based assays, either cationic lipids or microinjection was used to facilitate delivery of the oligonucleotide to the cytoplasm of the cell.

Demonstrating that the oligonucleotide is producing a pharmacological effect by an antisense mechanism of action *in vivo* is more difficult than in cell-based assays. Nevertheless, studies are being published, demonstrating pharmacological activity of oligonucleotides in *in vivo* models strongly supporting an antisense mechanism of action in which many of these same criteria were applied [3, 47–56]. If the studies are examined in aggregate, it is hard not to conclude that oligonucleotides are capable of inhibiting gene expression *in vitro* and *in vivo* by an antisense mechanism of action.

POTENCY. How much can the affinity of an oligonucleotide be improved over natural DNA or RNA and will this improvement translate to improved potency in pharmacological assays? This is another area in which application of medicinal chemistry has been quite successful [80]. There are a number of modifications that exhibit greater affinity than oligodeoxynucleotides for RNA, including various sugar modifications [14, 15, 81–83], heterocyclic modifications [76, 84–86], phosphoramidates [21], phosphate replacements [87–89], and sugar phosphate replacements [23]. In that potency in cellular based assays and *in vivo* is dependent on factors in addition to target affinity, the increase in affinity does not always translate to increase in potency in pharmacological assays. In many cases, the oligonucleotides need to be further modified with phosphorothioate linkages or other backbone modifications to protect from nuclease degradation. One concern with higher affinity oligonucleotides was that they would lose specificity [90]; however, this has not been borne out. In cases where it has been examined, the modified oligonucleotide has exhibited equal or greater selectivity for its Watson–Crick hybridization partner [91].

Toxicology

WILL OLIGONUCLEOTIDES HYBRIDIZE TO NON-TARGET RNA MOLECULES? In theory an oligonucleotide 11–15 bases in length should uniquely hybridize to a given mRNA, while an oligonucleotide 15–19 bases in length should hybridize to a unique DNA sequence depending on the A + T and G + C content [92]. These conclusions were based upon the assumption that the oligonucleotide would have equal access to all sites on the target RNA or DNA and whatever terminating mechanisms are utilized, they do not exhibit any sequence dependence. Both of these assumptions have proven to be incorrect. Multiple experiments have indicated that not all sites on a target RNA are equally accessible to the oligonucleotide either *in vitro* or *in vivo* [24, 63, 70, 72, 79, 93–96]. In addition, terminating mechanisms appear to be highly dependent upon the context of the sequence within the RNA, especially for oligonucleotides that do not utilize RNase H [24, 63, 67]. Therefore, it is difficult to predict what the chances are that a given oligonucleotide will hybridize to a non-target RNA and affect expression. In our experience this has not been a major issue. It is probably more important to optimize length of the oligonucleotide based upon potency, rather than selectivity for target RNA. This will vary depending on the chemistry used. For phosphorothioate oligodeoxynucleotides, optimal length may range from 15 to 21 bases in length, while higher affinity analogs would allow use of shorter oligomers [79].

INTERACTION WITH OTHER NON-TARGET MOLECULES. It should be appreciated that oligonucleotides, like any other molecules, are capable of interacting with non-target molecules [5, 6, 97–102]. In some cases, these interactions can be quite sequence specific, such as aptamers [103–105], while in other instances the oligonucleotides interact with molecules in a sequence-independent manner. Interaction with non-nucleic acid targets can contribute to their overall pharmacological activity, at times making it difficult to interpret which activity is due to the antisense effect and which is due to a non-antisense effect. Incorporating multiple controls into the experiment is important in drawing any conclusions.

There are a variety of proteins with which oligonucleotides interact in an apparently sequence-independent manner, including DNA polymerases, laminin, and CD4 [106, 107]. In many cases phosphorothioate oligodeoxynucleotides were more potent than phosphodiester oligodeoxynucleotides, suggesting that the sulfur substitution enhances these interactions. From a toxicological perspective, these interactions have not at this time been proven to result in untoward effects. The primary acute toxicities that are a concern are interaction with proteins in the clotting cascade, resulting in an anti-coagulant effect, and interaction with a protein or proteins resulting in complement activation [3]. In both cases it is unknown which proteins the oligonucleotides are interacting with to produce these effects, although candidate proteins have been identified.

Recently, several papers have been published demonstrating that pharmacological activities originally assumed to be due to an antisense effect of the oligonucleotide were, in fact, due to interaction with proteins [100–102, 107, 108]. In many cases, this activity can be attributed to the presence of a consecutive series of guanine residues in the oligonucleotide, which are capable of forming a four-

stranded structure termed a G quartet [109], although it has not been demonstrated conclusively that these oligonucleotides do form such a structure. Oligonucleotides that contain three or more guanine residues in a row can also hybridize to an RNA target, complicating interpretation of data generated from such an oligonucleotide. A second sequence motif that has been demonstrated to produce pharmacological activity by a non-antisense mechanism of action is a CpG motif in which the CG residues are flanked by two purines on the 5′-end and two pyrimidines on the 3′-end [102]. Oligonucleotides containing such motifs have been shown to be very potent B lymphocyte mitogens and activators of NK cells, although the latter activity appears to be more restricted [110]. In our experience, all phosphorothioate oligodeoxynucleotides examined will produce some degree of immune stimulation. With the proper sequence context, the oligonucleotide can produce very profound immune stimulation [111]. Rodents appear to be more sensitive to this effect than primates [112].

ECONOMICS. Will oligonucleotide synthesis be commercially viable for human therapeutics? For treatment of disease with a high morbidity and a large unmet medical need the markets would support an antisense oligonucleotide; however, for markets with low morbidity or a met medical need it would be questionable whether the economics would support an antisense oligonucleotide, or many other types of drugs for that matter. At this time it is difficult to predict what the full burden of costs will be for the manufacture of a phosphorothioate oligodeoxynucleotide at the time of commercialization of the first systemically administered oligonucleotide. In 1990, it was estimated that the actual cost of synthesizing a 28-mer phosphorothioate oligodeoxynucleotide was $42,700 per gram [113]. Improvements in process research and cost reductions associated with scale have dramatically reduced the costs of manufacture approximately 40-fold compared with what they were in 1990. It is anticipated that costs can be reduced by another 8- to 10-fold upon implementation of processes required to manufacture at the ton per year scale. Although this cost may be high compared with that of traditional small molecules, it is similar to or even cheaper than other biotechnology products and is commercially feasible for many clinical indications.

ANTISENSE OLIGONUCLEOTIDES: CURRENT ISSUES

Hopefully it is apparent from the above discussion that most of the early concerns about antisense oligonucleotides have been addressed and that there have not been any issues that have arisen preventing application of the technology either as a research tool (if experiments are properly controlled) or, more importantly, as a therapeutic agent. The largest body of information is known about first-generation phosphorothioate oligodeoxynucleotides. Large-scale synthesis of phosphorothioate oligodeoxynucleotides is feasible, they appear to be well tolerated at therapeutically relevant doses, they produce predicted pharmacological effects, and they are broadly distributed to tissues. In fact, it is possible that a first generation phosphorothioate oligodeoxynucleotide will be on the market before the year 2000. Although phosphorothioate oligodeoxynucleotides appear to be acceptable as drugs or research tools, there are several properties that can be improved for broader applicability. As discussed above, second and third generation oligonucleotide chemistries are already demonstrating improved properties, as are some advanced formulations. Following is a brief discussion of some of the limitations of phosphorothioate oligodeoxynucleotides or current modifications. Whenever possible, solutions that are in hand or appear to be well on their way to solving the issue are highlighted.

Pharmacokinetics

DELIVERY ROUTES. Current applications of oligonucleotides utilize parenteral dosage forms. While this route of administration will be acceptable for the treatment of many diseases, alternate dosage forms that are more convenient for the patient will expand the application of antisense oligonucleotides. Agrawal and colleagues [114] published some very intriguing work in which they demonstrated that phosphorothioate 2′-O-methyl oligonucleotides exhibit enhanced stability to nucleases in the gastrointestinal tract and enhanced oral bioavailibility. Extension of these observations by either additional chemical modifications or formulations may further improve oral bioavailibility, so that it is not unrealistic to envisage oral dosage forms for oligonucleotides. In addition, investigating other non-parenteral routes of administration may provide additional opportunities.

TISSUE DISTRIBUTION. Although phosphorothioate oligodeoxynucleotides are broadly distributed to many peripheral tissues, it may be desirable either to selectively target a tissue with an antisense oligonucleotide or to target tissues that do not achieve high concentrations, such as brain or even lung. There are multiple ways this might be achieved, including chemical modifications and formulations. In addition, it may be desirable to direct the oligonucleotide to specific cell types in the tissue or make more of the oligonucleotide within a tissue available for hybridization to target mRNA. Numerous studies have been published demonstrating selective targeting of tissues or cell types within tissues for other types of agents using antibodies, peptides, carbohydrates, or vitamins demonstrating technical feasibility. It still remains to be determined whether these types of approaches will be commercially viable in terms of enhancing delivery of antisense oligonucleotides.

Pharmacology

POTENCY. There are at least three mechanisms by which the potency of antisense oligonucleotides can be improved *in vivo*: increase affinity for its receptor, decrease non-receptor interactions, or increase efficiency for delivery to the subcellular compartment in which the receptor is localized. As discussed above, there are numerous modifications that enhance affinity for the target RNA in the cell, demonstrating improved activity in cell-based assays. Some of these modifications also demonstrate decreased interaction with various proteins, simultaneously accomplishing two goals. Enhancing delivery to the intracellular target is still an area in which little progress has been made. Preliminary studies suggest that cholesterol conjugation to oligonucleotides improves delivery to target RNA [115, 116]. In cell culture based experiments, cationic lipid formulations have been shown to facilitate intracellular delivery of charged oligonucleotides to the target RNA. However, the utility of these formulations for enhancing intracellular delivery of oligonucleotides *in vivo* has yet to be demonstrated, although they will significantly alter the tissue distribution [117]. Ultimately, I feel that additional improvements will be forthcoming.

MORE EFFICIENT IDENTIFICATION OF ACTIVE OLIGONUCLEOTIDE. As discussed previously, numerous studies have demonstrated that not all target sites on a mRNA are equally accessible. This is an issue not only for phosphorothioate oligodeoxynucleotides, but also for all oligonucleotides. Currently, high affinity target sites on RNA are identified empirically through screening multiple oligonucleotides. In that it is not economical to screen for all possible oligonucleotides, it is never known if the highest affinity oligonucleotide possible has been identified. It would be highly desired to improve on this method, ideally through predictive computer algorithms. However, because current understanding of RNA structure and accessibility inside a cell is very limited, it is unlikely that this will be forthcoming in the next 5 years. Other methods for identifying target sites on RNA, such as combinatorial approaches or arrays [95, 118], may provide solutions in the near term.

Toxicology

CLEARANCE FROM NON-TARGET TISSUES. Although phosphorothioate oligodeoxynucleotides have the desirable property of being stable towards serum and cellular nucleases, in some cases this stability may ultimately produce a detrimental effect. Although there are no published studies demonstrating that this is a problem, it could be rationalized that if the rates of tissue clearance from a non-target tissue are slower than the rates of clearance from a target tissue, accumulation of oligonucleotide in non-target tissue would occur when dosed optimally for the target tissue. In particular, accumulation in kidney is a concern in that clearance rates from kidney may be slower than from other tissues [112, 119]. Both medicinal chemistry modifications and formulations have shown potential promise to change the tissue distribution away from the kidney.

CHRONIC IMMUNE STIMULATION. Although immune stimulation is much more pronounced in rodents than in primates, there is some concern about chronic, low-level stimulation of the immune system. Alternatively, for some immune-mediated disorders, the oligonucleotide, at clinically relevant doses, may prime the immune system to respond to other stimuli. In that modified oligonucleotides have been demonstrated to abrogate this immune stimulation [102, 120, 121], this should be less of an issue with newer oligonucleotides.

INTERACTION WITH PLASMA PROTEINS. Currently there are two types of plasma protein interactions that are a concern for phosphorothioate oligodeoxynucleotides: interaction with protein components of the clotting cascade, resulting in anti-coagulant effects, and interaction with proteins resulting in complement activation. In both cases, the critical protein component with which the oligonucleotide interacts has not been identified. There are a number of suspected candidates, but more information is needed to confirm that interaction with the protein produces the undesirable effect. Several sugar and phosphate backbone modified oligonucleotides have been demonstrated to exhibit fewer anti-coagulant effects and less potential for inducing complement. At the same time, these same modifications also increase potency.

It should be pointed out that interaction with plasma proteins may not be an entirely undesirable property of the oligonucleotide. Interaction with plasma protein likely prevents filtration of unbound oligonucleotide in the kidney and excretion into the urine. Furthermore, interaction with plasma proteins may facilitate movement out of the vasculature through transcytosis and may enhance cellular uptake. If the binding to plasma proteins is low affinity [26, 122], then the oligonucleotide can partition off the plasma proteins and ultimately bind the target RNA.

METABOLISM OF MODIFIED NUCLEOSIDES. There is a rich literature in the use of modified nucleosides and nucleotides for anti-viral and anti-cancer agents, demonstrating that modified nucleosides can be cytotoxic. Therefore, there should be some concern that some of the oligonucleotides incorporating modified nucleosides or internucleosidic linkage may exert unwanted toxicities through release of these modified residues upon degradation. The oligonucleotide would, in essence, serve as a prodrug for the release of cytotoxic nucleotides [123].

FUTURE OF ANTISENSE OLIGONUCLEOTIDES

Is the glass half full or half empty? I feel that the technology is at a transition. There have been many lessons learned in how to utilize the technology and what the limitations are.

With careful selection of antisense oligonucleotides and proper controls it is possible to demonstrate a reduction in target gene expression by an antisense mechanism of action both *in vitro* and *in vivo*, validating the concept. With the explosion of information coming from genome sequencing efforts, a method for rapidly identifying the biological function and validating interesting targets for larger drug discovery efforts will be needed. Antisense oligonucleotides are the most logical tool for answering these questions. With properly conducted experiments, antisense oligonucleotides should provide valuable information in this regard. Newer oligonucleotide chemistries provide greater signal-to-noise ratios in the *in vitro* assays. It is clear that no single oligonucleotide modification will solve all the issues the technology faces; therefore, it is important to have a number of chemical modifications available for application to specific projects. These modifications can be used in a matrix approach in which the oligonucleotide can be tailored for specific needs.

In addition, the lead oligonucleotides also have the potential for progressing on to the clinic. One advantage that antisense oligonucleotides have in this regard is that chemical synthesis, preclinical pharmacokinetics, and to a large extent toxicology are modular from molecular target to molecular target. Once an investment has been made for a specific compound, the costs incurred for additional compounds against additional molecular targets are considerably less. How broadly useful antisense oligonucleotides will be for the treatment of human diseases is still an unanswered question. An oligonucleotide is already benefiting patients with CMV retinitis.* Preliminary results from other studies are encouraging as well. As with any other drug that enters the clinic, it is likely there will be many more failures than successes. It is unrealistic to expect otherwise, as there are many factors, in addition to technological issues, which control the success of a drug. However, it is likely that antisense oligonucleotides will be a part of the pharmacopoeia in the future, providing benefit to patients. Therefore, it is my perspective that the glass is actually more than half full and there is a promising future for antisense technologies.

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